University of Alberta

Delivery of Lipoprotein Cholesterol to Distinct Metabolic Pools in Hepatoma Cells

by

Eric M. Huang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Biochemistry

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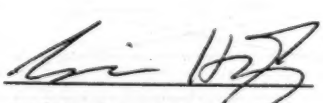

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DELIVERY OF LIPOPROTEIN CHOLESTEROL TO DISTINCT METABOLIC POOLS IN HEPATOMA CELLS submitted by ERIC M. HUANG in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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Abstract

Cholesterol is carried through the circulation by high-density and low-density lipoproteins (HDL and LDL, respectively). Disturbances in homeostatic mechanisms that control plasma cholesterol levels can lead to disease such as atherosclerosis. Elevated levels of LDL is a major risk factor for the development of atherosclerosis, whereas elevated HDL levels helps to protect against development of the disease. The difference is thought to lie in their metabolism by the liver. Three major enzymes that are important in hepatic cholesterol metabolism are 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGR), the rate-limiting enzyme in cholesterol biosynthesis; acyl-Coenzyme A: cholesterol acyl transferase (ACAT), the enzyme that catalyzes the esterification of cholesterol for storage; and cholesterol 7alpha-hydroxylase (cyp7a) the rate-limiting enzyme involved in the catabolism of cholesterol to bile acids. The overall goal of the thesis was to better understand the metabolism of HDL- and LDL-derived cholesterol in the rat hepatoma cell line McArdle RH7777. The experiments designed demonstrate that cholesterol delivered by HDL and LDL are delivered to different sub-cellular localizations. HDL- and LDL-derived cholesterol was shown to differ in its ability to down-regulate *de novo* cholesterol biosynthesis. In addition, the results suggest that LDL-derived cholesterol is more accessible to ACAT for esterification whereas, HDL-derived cholesterol is suggested to be more accessible to cyp7a for conversion to bile acids. Together, the results strongly suggest that HDL- and LDL-derived cholesterol is targeted to distinct metabolic pools.

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Abbreviations

ACAT	acyl-coenzyme A:cholesterol acyltransferase
ABCA1	adenosine triphosphate-binding cassette A1
ApoA-I	apolipoprotein A-I
ApoB	apolipoprotein B
ApoC-II	apolipoprotein C-II
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BAR	bile acid receptor
BSEP	bile salt export pump
C15.1	McArdle RH7777 stably expressing cholesterol 7alpha-hydroxylase
CA	cholic acid
CDCA	chenodeoxycholic acid
CE	cholesteryl ester
CEH	cholesterol ester hydrolase
CETP	cholesteryl ester transfer protein
Ci	curie
cMOAT	canalicular multispecific organic anion transporter
Co-A	Coenzyme A
CS	calf serum
Cyp7a	cholesterol 7 alpha-hydroxylase
DMEM	Dulbecco's modified Eagle's medium
DTPA	diethylenetriaminepentaacetate
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N',N'-tetra acetate
ER	endoplasmic reticulum
FBS	fetal bovine serum

FXR	farnesoid X receptor
HDL	high-density lipoprotein
HEK 293	human embryonic kidney 293 cells
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HRP	horseradish peroxidase
HSF	human skin fibroblast
IDL	intermediate density lipoprotein
LCAT	lecithin-CoA:cholesterol acyltransferase
LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
LFP	lipoprotein-free plasma
LPDS	lipoprotein deficient serum
LPL	lipoprotein lipase
LRP	LDL-R related protein
LXRα	liver X receptor alpha
M	molar
MDA	malondialdehyde
mM	millimolar
mg	milligram
ml	millilitre
μM	micromolar
McA	McArdle RH7777
MRP-2	multidrug-resistance associated protein-2
PBS	phosphate-buffered saline
PC	phosphatidyl choline
PL	phospholipid
PMSF	phenylmethylsulfonyl fluoride
PKA	protein kinase A
PS	phosphatidyl serine

PSL	phosphor-stimulated light units
RCT	reverse cholesterol transport
S1P	site-1 protease
S2P	site-2 protease
SCAP	SREBP cleavage activating protein
SCP-2	sterol carrier protein-2
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMC	smooth muscle cell
S-pgp	sister of P-glycoprotein
SR-BI	scavenger receptor, class B, type I
SRE	sterol-responsive element
SREBP	SRE-1 binding protein
TAG	triacylglycerol
TBARS	thiobarbituric acid reactive substances
TLC	thin-layer chromatography
VLDL	very low-density lipoprotein
UC	unesterified cholesterol

Chapter 1: Introduction

1.1. Atherosclerosis

Atherosclerosis is a major contributor to heart disease and stroke and is the leading cause of death in westernized societies (reviewed in Lusis, 2000). This disease is characterized by the progressive accumulation of lipids and fibrous elements in the large arteries (Lusis 2000). An elevated level of cholesterol in the plasma, primarily carried by low-density lipoproteins (LDL) plays a key role in the development of atherosclerosis. Elevated plasma levels of LDL can result from a variety of conditions ranging from genetic background to environmental conditions (diet) or a combination of both (Lusis 2000).

Atherosclerotic lesions typically develop in the aorta and then progress to the coronary arteries and cerebral arteries during the advanced stages of the disease (Lusis 2000). The accumulation of LDL in the sub-endothelial matrix of the artery wall marks the beginning of the development of the atherosclerotic lesion. Accumulation of LDL leads to the recruitment of monocytes to the surface of the endothelium. The monocytes transmigrate across the endothelial monolayer into the intima where they differentiate into macrophages and begin to take up lipoproteins. Inflammation of the endothelium leads to the further recruitment of monocytes into the intima. The macrophages in the arterial wall take up oxidized LDL in a non-feedback controlled manner *via* scavenger receptors. This leads to massive accumulation of intracellular cholesterol forming cholesterol-laden macrophages and formation of 'foam cells'. These foam cells accumulate in the intima, creating a lesion known as the 'fatty streak'.

In time, the death of foam cells and accumulation of lipid-rich, necrotic debris leads to the inflammation of the artery wall and recruitment of smooth muscle cells (SMCs). SMCs secrete fibrous elements and the atheroma becomes a fibrous lesion with a fibrous cap consisting of SMCs and extracellular matrix. As the fibrous plaques grow in size, calcification and ulceration can occur at the luminal surface of the lesion. Hemorrhage due to rupture or erosion of the lesion can lead to the formation of a thrombus or blood clot. The release of the thrombus may cause an occlusion of a coronary or cerebral artery, ultimately resulting in myocardial infarction or stroke, respectively (Lusis 2000).

Because elevated levels of cholesterol in the plasma are a major contributing factor in the development of atherosclerosis, understanding the homeostatic mechanisms involved in the elimination of cholesterol from the body becomes important. By understanding the mechanisms behind the catabolism and elimination of cholesterol from the body, one can then develop possible ways of treatment to lower plasma cholesterol and thus risk for developing atherosclerosis.

1.2. Cholesterol and the Liver

Cholesterol is an important biological molecule serving as a key structural component of membranes, modulating permeability and fluidity. Cholesterol is also important for cell growth and differentiation, and is the steroid precursor for bile acids, steroid hormones and vitamin D (Edwards 1996).

The liver plays a pivotal role in the maintenance of total body cholesterol homeostasis. It is a major site for *de novo* biosynthesis of cholesterol and plays a major role in the secretion of lipoproteins, thus providing a source of lipids for the peripheral tissues (Glickman 1994). The liver also plays an important role in the disposal of cholesterol from the body. There are two major output pathways for the elimination of cholesterol from the body, both of which occur exclusively in the liver. The first is the conversion of cholesterol into bile acids for secretion, and the second is the direct secretion of cholesterol into bile (Russell 1992). The regulation of cholesterol homeostasis in the liver is tightly regulated. Perturbations in these homeostatic mechanisms can lead to elevated plasma levels of cholesterol, and subsequently, to atherosclerosis.

1.3. Cholesterol metabolism in the hepatocyte

1.3.1. *De novo* biosynthesis of cholesterol

The liver is a major site for *de novo* biosynthesis of cholesterol. The biosynthesis of cholesterol from acetyl-CoA involves approximately 30 distinct enzymatic steps (Rilling 1985), (Vlahcevic 1994). The rate limiting enzyme of the pathway, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (HMGR) is located in the smooth endoplasmic reticulum (SER) and converts HMG-CoA into mevalonate (Vlahcevic 1994). Mevalonate then undergoes a number of enzymatic steps yielding a variety of isoprenoid intermediates, eventually producing cholesterol (Vlahcevic 1994).

The details of the intracellular trafficking of newly synthesized cholesterol still remains a subject of debate. Klansek, *et al.* suggest that nascent cholesterol is preferentially utilized by the enzyme acyl-Coenzyme A:cholesterol acyltransferase (ACAT) to form CE (Klansek 1996). This is thought to be due to the spatial proximity of ACAT and enzymes of cholesterol biosynthesis. In contrast to these findings, other groups have reported that newly synthesized cholesterol rapidly appears at the plasma membrane (Smart 1998), (Phillips 1998). However, the mechanism by which this occurs is not yet clear. There is evidence to suggest that newly synthesized cholesterol travels from the site of synthesis (ER) to the plasma membrane by vesicular transport that bypasses the *trans*-Golgi Network (TGN; (Kaplan 1985), (Urbani 1990)). Other groups suggest that newly synthesized cholesterol is rapidly transported to the plasma membrane by a non-vesicular mechanism (Fielding 1996a), (Fielding 1997).

1.3.1.1. Regulation of cholesterol biosynthesis

The rate of *de novo* biosynthesis of cholesterol is tightly regulated. This is achieved by feedback mechanisms that sense the intracellular levels of cholesterol and modulate the transcription of genes encoding enzymes of cholesterol biosynthesis and uptake from plasma lipoproteins (Brown 1999). This regulation occurs by the sterol regulatory element-binding protein (SREBP) family of membrane transcription factors (Brown 1997).

SREBPs are proteins located in the ER and nuclear membranes. They are approximately 125kDa proteins and have three main domains. The NH_2-terminal domains of SREBPs are transcription factors belonging to the basic helix-loop-helix leucine zipper family of transcription factors. The central domain contains two transmembrane domains, linked by a short (approximately 31 amino acid) domain, found in the lumen of the ER or Golgi. The third domain is the carboxy-terminal regulatory domain.

Newly synthesized SREBP is inserted into the nuclear and ER membranes. In sterol-depleted cells, the 68kDa NH_2-terminal domain is released from membranes following two sequential proteolytic cleavages, both of which must occur in the proper order (Sakai 1996). Proteolytic cleavage begins with Site-1 Protease (S1P), separating SREBP into two halves, both remaining membrane bound (Sakai 1996). Site-2 Protease (S2P) then cleaves the NH_2-terminal intermediate, releasing the NH_2-terminal fragment at a site just within the membrane spanning domain. Recent studies report that S2P cannot act until the two halves of SREBP have been separated by S1P (Sakai 1996). The 68 kDa, trancriptionally active NH_2-terminal fragment is translocated to the nucleus where it binds to sterol-response element (SRE) of multiple genes encoding enzymes of unsaturated fatty acid biosynthesis, triacylglycerol (TAG) biosynthesis, lipid uptake and cholesterol biosynthesis (Horton 1999). SREBP has been demonstrated to target HMG-CoA synthase, HMGR, farnesyl diphosphate synthase, and squalene synthase (Edwards 1998). Under conditions when cells

are cholesterol-loaded, cleavage by S1P is blocked, preventing the release of the NH_2-terminal fragment.

Recently, the SREBP cleavage-activating protein (SCAP) has been identified as a regulatory protein necessary for cleavage at Site-1 (Hua 1996). SCAP has 2 distinct domains: a 730 amino acid region of alternating hydrophobic and hydrophilic residues forming 8 membrane spanning helices, and a second region that anchors SREBP to the ER membrane (Nohturfft 1998). Within the membrane spanning domain lies a segment that comprises the 'sterol-sensing domain'. It is postulated that this sterol-sensing domain may interact with cholesterol within the membrane, but the function of this domain remains unknown (Brown 1999).

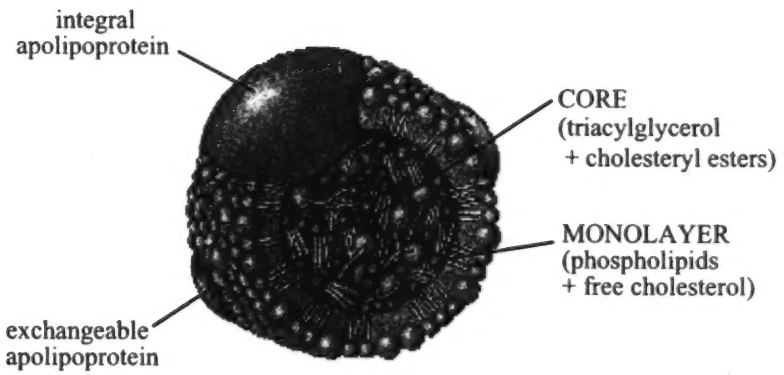
Within cells, SCAP is found tightly complexed with SREBP (Nohturfft 1998). Studies have demonstrated that SCAP functions as a chaperone protein that transports the 125 kDa SREBP precursors from the ER to a compartment that is accessible to S1P (DeBosse-Boyd 1999), (Sakai 1998). It is proposed that the SCAP/SREBP complex is the true substrate for S1P (Brown 1999).

There are three forms of SREBP: SREBP-1a, -1c and 2. SREBP-1a and 1c are a result of alternate splicing and the use of alternate promoters, whereas the SREBP-2 gene encodes a single protein with 50% identity to SREBP-1 (Brown 1997). An important difference between these SREBPs lies in their preference for activating different genes. SREBP-2 preferentially activates genes involved in cholesterol biosynthesis over fatty acid synthesis, whereas SREBP-1 preferentially activates genes involved in fatty acid synthesis over cholesterol

synthesis (Brown 1997). Another important difference between SREBP-1 and –2 is that proteolytic cleavage can be regulated independent of each other (Sheng 1995). However, these mechanisms of regulation remain unclear.

1.4. Lipoprotein metabolism

Lipids, found mainly in the form of TAG, cholesteryl ester (CE), unesterified cholesterol (UC) and phospholipid (PL) are transported through the circulation in the form of plasma lipoproteins. A diagram depicting the general structure of a mammalian lipoprotein particle is shown in Figure 1-1. A lipoprotein particle generally consists of a hydrophobic core of neutral lipid (TAG and CE) surrounded by a monolayer of amphiphilic lipids (phospholipids and cholesterol) and some specific apolipoprotein(s). This packaging allows for the solubilization and transport of hydrophobic and amphiphilic molecules through aqueous medium (Davis 1996). The major species of lipoproteins are distinguished by their specific buoyant densities when plasma is subjected to isopycnic gradient ultracentrifugation. The major classifications are as follows: chylomicrons (ρ<1.000 g/ml), very low-density lipoproteins (VLDL; ρ1.000-1.006 g/ml), intermediate density lipoproteins (IDL; ρ=1.006-1.019 g/ml), low density lipoproteins (LDL; ρ=1.019-1.063 g/ml) and high-density lipoproteins (HDL; ρ=1.063-1.25 g/ml) (Davis 1996). There are two major classes of



ApoB-containing lipoproteins	**Density**
• Chylomicrons	ρ<0.95 g/ml
• very low-density lipoproteins (VLDL)	ρ=0.95-1.006 g/ml
• intermediate-density lipoproteins (IDL)	ρ=1.006-1.019 g/ml
• low-density lipoproteins (LDL)	ρ=1.019-1.063 g/ml
ApoA-containing lipoproteins	
• high-density lipoproproteins (HDL)	ρ=1.063-1.21 g/ml

Figure 1-1. General Structure of Mammalian Lipoprotein Particle

There are two major classes of lipoproteins: apoA- and apoB-containing lipoproteins. The major species of lipoproteins are distinguished by their buoyant densities when plasma is subjected to isopycnic gradient ultracentrifugation. Adapted from reference (Grundy 1990).

lipoproteins: apolipoprotein (apo) B-containing lipoproteins (chylomicrons, VLDL, IDL and LDL) and apoA-containing lipoproteins (HDL).

1.4.1. Apolipoproteins

Apolipoproteins primarily serve as structural components by aiding in particle stabilization. There are two classes of apolipoproteins: integral and exchangeable apolipoproteins. The integral apolipoproteins include apoB100 and apoB48. These apolipoproteins are large and in addition to providing structure for the lipoprotein particle, they also confer ligand specificity to cell surface receptors (reviewed in Davis, 1996).

Exchangeable apolipoproteins, such as apoA-I, apoA-II, apoC-II and apoE are smaller proteins. Their exchangeable nature confers the ability to be transferred among lipoprotein particles, allowing for modulation of ligand specificity to cell surface receptors as well as enzyme activation (reviewed in Fielding, 1996).

1.4.1.1. ApoB-containing lipoproteins

ApoB-containing lipoproteins are assembled as VLDL and chylomicrons. The difference between these two lipoproteins lies in respective their sites of synthesis and isoform of apoB. VLDL particles are synthesized in the liver, with apoB100, a 4536 amino acid protein. Chylomicrons are synthesized in the

intestine post prandially with apoB48, a truncated protein comprising the first 48% of apoB100 containing 2152 amino acids (Powell 1987), (Chen 1987).

Nascent apoB-containing lipoproteins are composed mainly of PL and TAG and apoB100 or apoB48. Once released into the plasma, the particles accumulate the apoC-II and apoE. The particle binds to and activates lipoprotein lipase (LPL) upon recognition of apoC-II, and TAG is rapidly hydrolyzed. The depletion of the TAG core by LPL results in a decrease in particle size and increase in particle density. A reduction in size reduces the particles' affinity for apoC-II. Dissociation of apoC-II reduces LPL's affinity for these particles and they are released back into circulation as remnant particles (Fielding 1996b). The dissociation of apoC-II is accompanied by the release of PL and UC into the circulation. These products contribute to the formation of the HDL particle (see Section 1.4.1.2.).

Chylomicron remnants are taken up by hepatocytes *via* the LDL-receptor (LDL-R) or the LDL-R related protein (LRP) upon recognition of apoE. As VLDL remnants decrease in size, they gradually become IDL and eventually LDL. LDL is internalized by the hepatocyte *via* LDL-R upon recognition of both apoB100 and apoE, as well as by LRP (Hussain 1999). A general diagram depicting the circulation and processing of apoB-containing lipoproteins is shown in Figure 1-2.

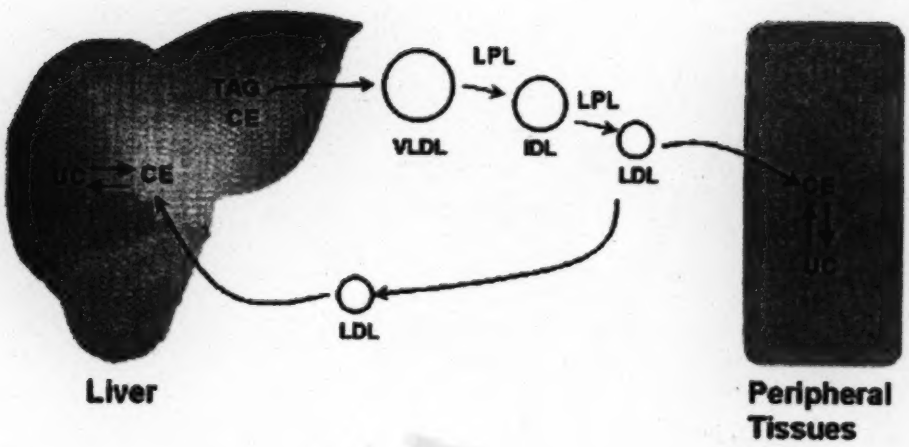


Figure 1-2. A general diagram illustrating the circulation and processing of apoB-containing lipoproteins.

ApoB-containing lipoproteins are assembled and secreted by the liver in the form of a TAG-rich VLDL particle. Lipoprotein lipase hydrolyzes the TAG causing a decrease in particle size and increase in particle density. VLDL forms IDL and eventually LDL. LDL can be taken up by the peripheral tissues and the liver by the LDL-receptor (LDL-R). Cholesterol that is delivered to the liver may be re-assembled and secreted as lipoproteins or eliminated from the body through the bile. Adapted from references (Oram 2001) and (Krieger 1999).

1.4.1.1.1. Metabolism of LDL-derived cholesterol

The LDL-R and LRP can mediate the internalization and complete degradation of the CE-laden LDL particle via receptor-mediated endocytosis (reviewed in Brown, 1986, Mukherjee, 1997, and Hussain, 1999). Figure 1-3 illustrates the general steps involved in LDL-receptor-mediated endocytosis. The process begins with the assembly of a clathrin-coated pit. Clathrin is a protein composed of three light and three heavy chains that form a three-legged structure called a triskelion. Triskelions then assemble into a basket-like convex framework of hexagons and pentagons on the cytoplasmic surface of the plasma membrane forming the coated pit (Kirchhausen 1993), (Schmid 1992). The formation of the clathrin-coated pit allows for the recruitment of ligand receptors, present on the plasma membrane. Upon binding of ligand, coated pit pinches off of the plasma membrane, internalizing the LDL-particle. Following internalization, the vesicle undergoes rapid uncoating of clathrin, which is recycled back to the plasma membrane for formation of a new coated pit. The uncoated vesicle containing the LDL-particle is then trafficked into the endosomal/lysosomal degradative pathway. A vacuolar H^+/ATPase acidifies the environment of the endosome (Al Awqati 1986), causing the dissociation of the ligand from its receptor, allowing for the receptor to be recycled back to the plasma membrane to participate in another round of endocytosis (Davis 1987), (Yamashiro 1987). LDL is subsequently delivered to the lysosome where apolipoproteins are hydrolyzed to their component amino acids and the

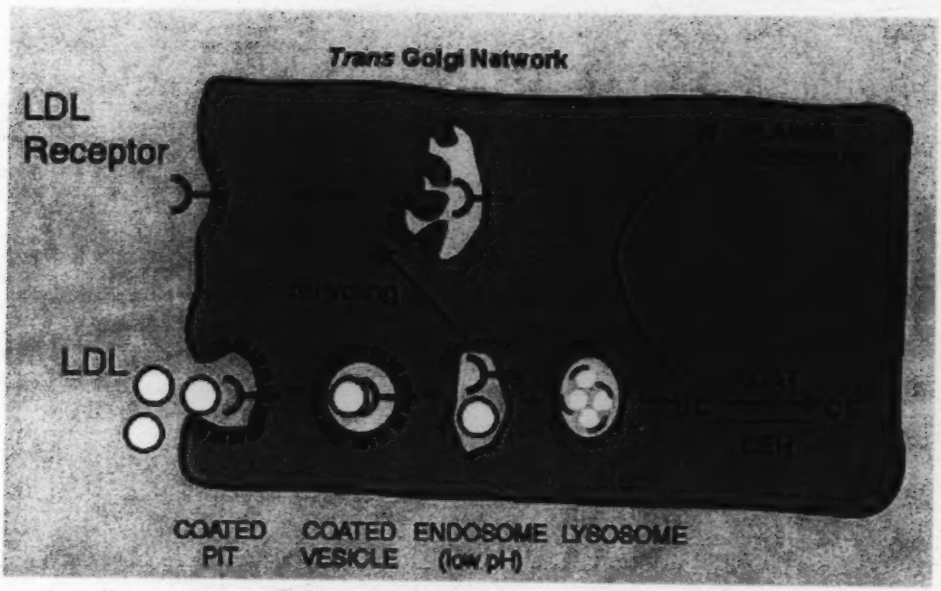


Figure 1-3: General diagram depicting the major steps in LDL-receptor-mediated endocytosis.

The LDL-receptor (LDL-R) is located on the plasma membrane in a clathrin coated pit. Upon binding to an LDL particle, the coated pit pinches off the plasma membrane forming a coated vesicle. The vesicle is un-coated and the clathrin returns to the plasma membrane forming a new coated pit. The vesicle enters the endosomal/lysosomal degradative pathway. A vacuolar H^+/ATPase acidifies the endosome causing the ligand to dissociate from the LDL-R. The LDL-R is then recycled back to the plasma membrane *via* the *trans*-Golgi network where it participates in another round of receptor-mediated endocytosis. The LDL particle is delivered to the lysosome where the apolipoprotein is hydoylzed to its component amino acids and CE hydrolyzed to UC. The UC is trafficked to the plasma membrane and also a substrate for ACAT for esterification. CEH catalyzes the hydrolysis of CE to UC. Adapted from reference (Krieger 1999)

CE core is hydrolyzed to UC by acid cholesterol ester hydrolase (Rhainds 1999), (Hui 1996).

The bulk of LDL-derived cholesterol (approximately two-thirds) has been suggested to be trafficked by vesicular transport to the Golgi, followed by transfer to the plasma membrane and subsequently to the ER (Neufeld 1998). Intralysosomal hydrolysis of CE by an acidic lipase has been reported to take less than 2 minutes (Brasaemle 1990). The overall transfer time from the clathrin-coated pit to the plasma membrane is postulated to be between 37 to 50 minutes (Brasaemle 1990), (Jefferson 1991), (Johnson 1990). The remaining one-third of LDL-derived cholesterol is thought to be transported from the lysosome to the ER in a vesicular pathway that bypasses the plasma membrane (Underwood 1998). This cholesterol has been shown not to mix with plasma membrane cholesterol (Underwood 1998) and is accessible to cholesterol regulatory proteins such as SREBP and ACAT (Neufeld 1996). This is confirmed by studies that demonstrate that LDL-derived cholesterol causes a reduction of HMGR expression (Saucier 1989), a reduction in LDL-R expression (Brown 1986), and activates expression of ACAT (Goldstein 1974). Furthermore, cholesterol delivered by this pathway is made available for esterification by ACAT (Li 2001). A small amount of LDL-derived cholesterol has also been shown to be transported directly from the lysosome to the ER via a non-vesicular, protein-mediated pathway, bypassing both the Golgi and plasma membrane. Sterol carrier protein-2 (SCP-2) has been postulated as a candidate for this protein

(Liscum 1999), (Schroeder 2001). However, the significance of this pathway remains to be elucidated.

1.4.1.2. ApoA-containing lipoproteins

The main apolipoproteins of HDL are apoA-I and apoA-II. In humans, apoA-I is synthesized by both the liver and intestine, and apoA-II by the liver. HDL begins as a lipid-poor apoA-I particle. The formation of the HDL particle largely occurs extracellularly and plays a key role in 'reverse cholesterol transport' (please refer to Section 1.4.2.). A diagram depicting the major steps of HDL-formation is shown in Figure 1-4. As mentioned in Section 1.4.1.1., the lipolysis of apoB-containing lipoproteins releases apoC-II, as well as PL and UC into the plasma. These products contribute to the formation of the HDL particle. Lipid-poor apoA-I particles secreted by the liver facilitate the efflux of unesterified cholesterol from peripheral cells (discussed in Section 1.4.2) to forming a discoidal, UC-rich HDL particle (Castro 1988), (Huang 1993). The discoidal HDL particle has a high affinity for lecithin-cholesterol acyltransferase (LCAT) which esterifies the accumulated UC to form CE. The CE packs into the centre of the mature, spherical HDL particle. HDL can then transport its cholesterol back to the liver.

The liver can internalize cholesterol from HDL by receptor-mediated endocytosis (described in Section 1.4.1.1.1.) and by selective lipid uptake (described in Section 1.4.1.2.1.). The larger, CE-enriched HDL particles may

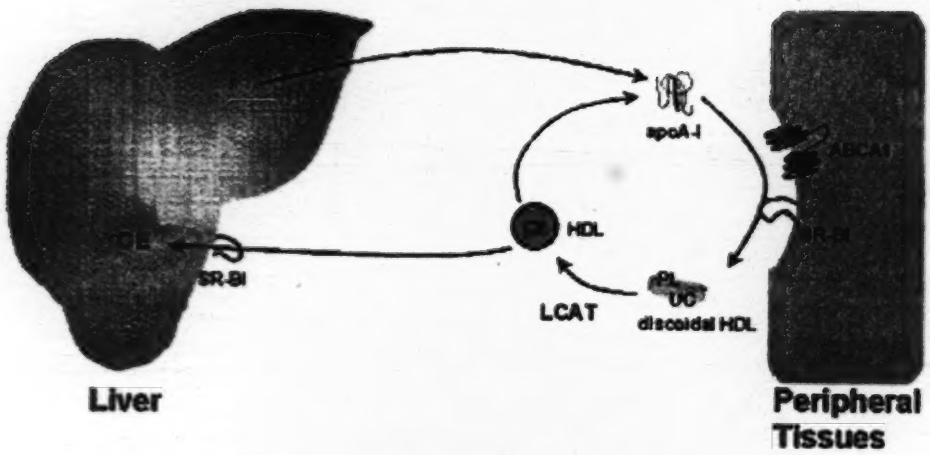


Figure 1-4. Diagram depicting the major steps in HDL formation

HDL formation occurs extracellularly and begins in the periphery. The liver secretes apoA-I. This particle serves as an acceptor for UC and PL effluxed from peripheral cells. This process involves ABCA1 and SR-BI. Lipidation of apoA-I with UC forms a discoidal HDL particle. Discoidal HDL has affinity for LCAT that functions to esterify the UC to CE. Esterification of UC forms a spherical CE-rich mature HDL particle. HDL can then return cholesterol to the liver *via* selective uptake by SR-BI. Removal of CE from HDL either by selective uptake regenerates a lipid-poor apoA-I particle that can participate in another cycle of cholesterol efflux. Adapted from reference (Oram 2001).

become associated with apoE and be internalized by LDL-R or LRP (Fagan 1996), (Ji 1997). However, it has recently been demonstrated that cholesterol from HDL can also be taken up by the liver without whole-particle internalization (Reaven 1995), (Reaven 1996), (Acton 1996), (Rigotti 1997), (Frolov 2000). In rats, the selective delivery of lipids, but not proteins, from HDL is believed to be an important mechanism for the transport of cholesterol to the liver, comprising approximately 65% of HDL-CE clearance (Krieger 1999). Scavenger receptor Class B, Type I (SR-BI), has been identified as a physiological receptor for HDL (Acton 1996) and mediates the selective uptake of cholesterol from HDL, as well as from other lipoproteins (Krieger 1999). The selective uptake of cholesterol from HDL regenerates a lipid-poor apoA-I particle, which can then participate in another round of cholesterol efflux from the periphery.

HDL-derived cholesterol can also be internalized by the liver by transfer of CE to other lipoproteins. This process is facilitated by cholesteryl ester transfer protein (CETP). In species that express CETP, CETP exchanges CE in HDL for TAG from circulating LDL (Tall 1998). The TAG-enriched HDL particle becomes a substrate for hepatic lipase, re-converting the HDL particle into a lipid poor apoA-I particle, where it resumes its function as an acceptor for cholesterol in the periphery (Fielding 1996b).

1.4.1.2.1. Metabolism of HDL-derived cholesterol

SR-BI interacts with HDL mediating the non-endocytic selective uptake of both HDL-UC (Frolov 2000), (Hauser 1998) and HDL-CE (Reaven 1995), (Reaven 1996). Selective uptake by SR-BI was shown to require the presence of caveolae/caveolin-1 (Smart 1998). Not surprisingly, SR-BI was demonstrated to localize primarily to caveolae (Babbit 1997).

Caveolae are described as morphologically distinct "flask-shaped" invaginations of the plasma membrane (Anderson 1998). Caveolae are enriched with cholesterol, sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, ceramide and diacylglycerol, and are characterized as being resistant to detergents such as Triton X-100 (Anderson 1998). Interestingly, caveolae contain 10% of plasma membrane cholesterol, yet comprise only 1% of the plasma membrane (Schroeder 2001). Caveolin-1 is the marker protein used to identify caveolae during cellular fractionation (Anderson 1998). Caveolin-1 is thought to stabilize caveolae (Fielding 2001) and has been shown to bind cholesterol and is thought to play an integral role in cholesterol uptake and intracellular cholesterol transport. It is interesting to note that caveolae are reduced or completely absent in immortalized cell lines and many cancer cells (Koleske 1995), (Lee 1998).

The exact mechanism of HDL-derived cholesterol trafficking remains unclear and is under active investigation. Following internalization by selective uptake, HDL-derived CE is thought to be delivered to the Golgi/ER *via* a vesicular route that bypasses the lysosome (Reaven 1996). The CE is hydrolyzed

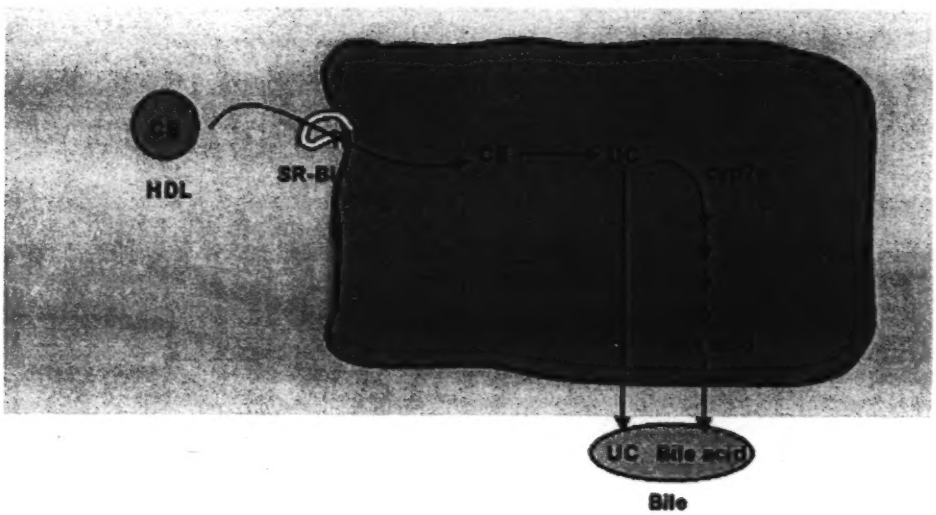


Figure 1-5: General diagram illustrating the selective uptake and trafficking of HDL-derived cholesterol

HDL-derived CE taken up by the cell *via* selective uptake. Following internalization, CE is hydrolyzed to UC extralysosomally. UC can then be utilized as a substrate for cyp7a for catabolism to bile acids or secreted directly into bile as UC. Adapted from reference (Krieger 1999).

extralysosomally by an enzyme distinct from the acid cholesterol ester hydrolase (Sparrow 1990). A diagram illustrating the internalization and trafficking of HDL-derived cholesterol is shown in Figure 1-5. Recent studies have implicated HDL-derived cholesterol to be preferentially utilized for bile secretion (please refer to Section 1.5.1.2.).

1.4.2. Reverse cholesterol transport

'Reverse Cholesterol Transport' (RCT) was a term introduced by Glomset in 1968 (Glomset 1968), describing a series of metabolic events resulting in the transport of excess cholesterol from the peripheral tissues to the liver for disposal (Fielding 1995). Because peripheral cells have a very limited ability to catabolize cholesterol, a mechanism must exist to facilitate the removal of excess cholesterol from these tissues.

The first step of RCT begins in the periphery with the efflux of cholesterol to acceptor particles such as serum albumin and apoA-I (Spady 1999). Three mechanisms have been proposed for cholesterol efflux. The first involves aqueous diffusion of cholesterol from membranes to acceptor molecules (Johnson 1991). The second involves protein-facilitated transport of cholesterol from caveolae to lipid-poor, apoA-I particle. The third model describes the active movement of cholesterol to lipid-poor apoA-I, requiring the hydroylsis of ATP. Each of these processes results in the generation of mature HDL-particles from lipid-poor HDL particles. HDL then delivers effluxed peripheral cholesterol back to the liver for elimination.

In the simple diffusion model, serum albumin serves as an acceptor for cholesterol as it moves down its concentration gradient (Fielding 1982), (Zhao 1996). The albumin-cholesterol serves as an intermediate, delivering cholesterol to circulating HDL. The concentration gradient is maintained by action of plasma LCAT, esterifying the incoming cholesterol (Fielding 2001).

The second model is the facilitated diffusion of cholesterol down a pre-existing concentration gradient. Recent studies suggest that caveolae contribute significantly to UC efflux. Introduction of caveolin-1 and caveolae in normal skin fibroblasts was paralleled by a corresponding increase in UC efflux (Fielding 1999). Introduction of caveolin antisense DNA , oxysterols or vanadate resulted in a comparable decrease in UC efflux (Fielding 2000), Recently, SR-BI was suggested to play a role in the efflux of cholesterol (Ji 1997). SR-BI is localized to caveolar domains of the plasma membrane (Babbit 1997), (Matveev 1999) and is shown to catalyze both selective uptake of lipids (discussed in Section 1.4.3.) and efflux of UC (Silver 2001). Increasing the expression of SR-BI up-regulated the efflux of cholesterol (Ji 1997). The acceptor molecule for UC efflux is thought to be lipid-poor apoA-I particles (discussed in Section 1.3.1.2.) (Castro 1988), (Huang 1993), eventually forming CE-rich, mature HDL through action of LCAT.

Active cholesterol efflux is thought to involve an ATP-binding cassette (ABC) transporter, ABCA1 (Oram 2000). ABCA1 is a member of the ABC superfamily of proteins that utilizes ATP as a source of energy to transport substrates between different cellular compartments and from the cell (Klein

1999), (Dean 2001)). Mutations in ABCA1 have been demonstrated to be the cause of Tangier disease and other familial HDL deficiencies (Oram 2000). These disorders are characterized by very low plasma HDL levels and reduced apoA-I levels (Marcil 1995). However, the role of ABCA1 in mediating active cholesterol efflux is controversial and under active investigation.

It was previously reported that a structurally and functionally intact ABCA1 transporter was required for binding of apoA-I (Lawn 1999), (Oram 2000). Furthermore, cholesterol efflux was thought to involve direct binding of apoA-I, suggesting that lipid-free apoA-I is the physiological substrate for ABCA1 (Wang 2000). In contrast, recent studies strongly suggest that apoA-I does not bind to ABCA1 directly (Chambenoit 2001). Interestingly, ABCA1 was reported to 'flop' phosphatidylserine (PS) from the inner leaflet to the exofacial leaflet of the plasma membrane (Marguet 1999), (Hamon 2000), despite phosphatidylcholine (PC) being the predominant PL effluxed to apoA-I. Furthermore, the removal of PS from the membrane was minimal (Fielding 2000), (Wang 2001). This suggested that the transfer of PC from the membrane was a secondary effect of the destabilization of the plasma membrane (Fielding 2001). In studies where caveolin-1 expression is inhibited with caveolin antisense DNA, UC, but not PL efflux was inhibited. In addition, there was generation of a PL-rich apoA-I complex (Fielding 2000). Taken together, these results suggest that UC efflux is neither the result of, nor required for, ABCA1 activity (Fielding 2001). It is therefore suggested that HDL formation is a two-step process: generation of the

initial apoA-I/PL complex by action of ABCA1, followed by the addition of UC to this activated complex (Fielding 2000), (Fielding 2001).

Following cholesterol efflux to HDL, HDL-CE is returned to and internalized by the liver by selective lipid uptake, mediated by SR-BI. The final steps of RCT involve the removal of cholesterol from the body.

1.5. Cholesterol elimination

The formation of bile acids is a process that exists exclusively in the liver. There are two major mechanisms that lead to the elimination of cholesterol from the body. The first is the catabolism of cholesterol into bile acids and the second is the direct canalicular secretion of cholesterol into bile (Russell 1992).

1.5.1. Bile acid biosynthesis

Two pathways exist for the breakdown of cholesterol to bile acids: the classical pathway and the alternative pathway (reviewed in (Agellon 2000), (Agellon 2002)). The following diagram illustrates the major steps in the classical and alternative bile acid biosynthetic pathways (Figure 1-6). The classic or neutral bile acid biosynthetic pathway begins with the hydroxylation at the C-7 position of the steroid ring of cholesterol, catalyzed by cholesterol 7 alpha-hydroxylase (cyp7a) yielding, 7α-hydroxycholesterol. This represents the first and rate-determining step of the classical bile acid biosynthetic pathway (Russell 1992). The

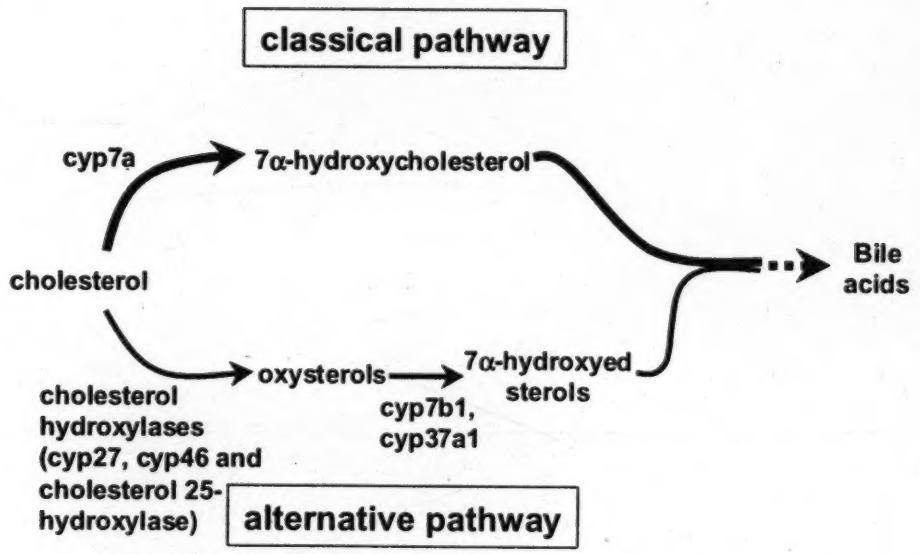


Figure 1-6: Diagram illustrating the major steps in the Classical and Alternative bile acid biosynthetic pathways.

The first and rate-limiting step of the classical pathway is catalyzed by cholesterol 7alpha-hydroxylase (cyp7a). This pathway occurs entirely in the liver. Cholesterol hydroxylases (Cyp27, sterol 27-hydroxylase; cyp46, cholesterol 24-hydroxylase; cholesterol 25-hyroxylase) facilitate the entry of cholesterol into the alternative pathway. The generated oxysterols are further 7α-hydroxylated by oxysterol hydroxylases cyp7b1 and cyp37a1, and the products enter into the latter steps of the classical pathway. Adapted from reference (Agellon 2002).

product of this reaction then undergoes subsequent enzymatic steps resulting in the saturation of the steroid nucleus and epimerization of the 3β-hydroxyl group. The addition of a 12α-hydroxyl group by sterol 12α-hydroxylase produces a precursor for cholic acid (CA). Alternatively, reduction of the steroid ring leads to the production of a precursor of chenodeoxycholic acid (CDCA). The mitochondrial enzyme sterol 27-hydroxylase (cyp27) then initiates side chain modification, ultimately leading to the formation of the primary bile acids: CA and CDCA (Russell 1992). Cyp27 also plays an important role in the alternative pathway (see below). In humans, the classical pathway produces approximately equal amounts of CA and CDCA. Following side chain oxidation and cleavage at the C-24 position, bile acid CoA:amino acid N-acyltransferase conjugates primary bile acids with glycine or taurine.

The alternative pathway for the synthesis of bile acids involves the production of oxysterols catalyzed by several sterol hydroxylases: sterol 27-hydroxylase (cyp27) (Cali 1991), cholesterol 25-hydroxylase (Lund 1998) and cholesterol 24-hydroxylase (cyp46) (Lund 1999). Nearly all 24-hydroxycholesterol originates from the brain, suggesting that its production is a major mechanism of removing excess cholesterol from this organ (Bjorkhem 2001). Cyp27, in addition to being expressed in the liver (Andersson 1989), is also expressed in a variety of extrahepatic tissues including the ovary (Su 1990), vascular epithelium (Reiss 1994), atherosclerotic plaques (Crisby 1997), macrophages (Babiker 1997) and fibroblasts (Zhang 1995). Because bile acid biosynthesis can only be completed in the liver, following the hydroxylation by

cyp27, 3β-hydroxy-5-cholestanoic acid is transported back to the liver, where bile acid biosynthesis is completed (Rosen 1998), (Schwarz 1997). It has been suggested that cyp27 may be involved in "reverse cholesterol transport" by converting the highly non-polar cholesterol into a more polar product for transport back to the liver for secretion (Bjorkhem 1994), (Babiker 1997).

The oxysterols generated by these sterol hydroxylases are subsequently 7α-hydroxylated by oxysterol hydroxylases distinct from cyp7a. Cyp7b1 oxysterol 7α-hydroxylase prefers 25- and 27-hydroxycholesterol (Agellon 2002) whereas cyp37a1 oxysterol 7α-hydroxylase prefers 24-hydroxycholesterol. Subsequent enzymatic steps result in sterol ring modifications similar to those in the classic pathway and ultimately result in side-chain cleavage to yield primary bile acids. In contrast to the classic pathway, the alternative pathway yields predominantly CDCA and contributes quantitatively little to overall bile acid biosynthesis except in certain pathophysiological conditions (Vlahcevic 1999).

1.5.1.1. Transcriptional regulation of cholesterol 7alpha-hydroxylase

Much of the study of cyp7a gene expression is at the level of gene transcription (Agellon 2002). Regulation of cyp7a involves a variety of biological molecules including oxysterols (Janowski 1999), (Lehmann 1997) and bile acids (Vlahcevic 1999). It has been demonstrated that oxysterols

mediate feed-forward induction of cyp7a gene expression, facilitated by the nuclear receptor liver X receptor alpha (LXRα) (Lu 2000). In contrast, bile acids mediate negative-feedback inhibition of cyp7a gene expression. The recently discovered bile acid receptor (BAR; also referred to as farnesoid X receptor, FXR) was demonstrated to mediate this response (Makishima 1999), (Parks 1999), (Wang 1999).

1.5.1.2. Regulation of bile acid biosynthesis by substrate availability

The two major input pathways of cholesterol are *de novo* biosynthesis and the internalization of cholesterol from circulating lipoproteins. From various *in vivo* and *in vitro* experiments, it is considered that cholesterol secreted as bile acids and biliary cholesterol is derived principally from plasma lipoproteins with only a minor contribution from newly synthesized cholesterol (Botham 1995), (Schwartz 1982), (Schwartz 1993).

The question of which lipoprotein species delivers to bile acid and biliary cholesterol secretion has long been investigated. Epidemiological studies demonstrate a strong inverse correlation between plasma HDL levels and risk of developing atherosclerosis (Gordon 1989). It has been proposed that reverse cholesterol transport occurs as a consequence of HDL facilitating the removal of excess cholesterol from the periphery and delivery to the liver for secretion into bile (Lusis 2000). There is a body of evidence strongly suggesting that UC carried by HDL be preferentially utilized for biliary cholesterol secretion.

(Schwartz 1978), (Bravo 1989). Studies involving the overexpression of SR-BI demonstrated the drastic reduction in plasma HDL levels (Kozarsky 1997), (Wang 1998) and parallel increase in biliary secretion of cholesterol (Kozarsky 1997), (Sehayek 1998), (Ji 1999). Recently, in a study using plant sterols as UC analogs, it was demonstrated that UC from HDL, but not from other lipoproteins is secreted directly into bile (Robins 1997). It has also been demonstrated that HDL-CE can be converted to bile acids (Bravo 1993), (Bravo 1994). In the latter study, the results suggest that a greater proportion of HDL-UC appeared as biliary cholesterol compared to HDL-CE from intravenously administered [^{3}H]HDL-UC or [^{3}H]HDL-CE.

The evidence presented above does not however, preclude the possibility that LDL-derived cholesterol may also deliver cholesterol to bile. Gene therapy studies report that overexpression of cyp7a resulted in dramatic decline in serum LDL cholesterol levels (Spady 1995), (Agellon 1997). Overexpression of cyp7a in mice lacking the LDL receptor was also successful in lowering plasma LDL levels (Spady 1998). In a study using primary hepatocytes fed β-VLDL and LDL, Post, *et al.* reported an upregulation of cyp7a activity, an effect not observed when fed HDL (Post 1999). In a study involving LDL apheresis in patients, Hillebrant, *et al.* observed a 26% decrease in plasma LDL paralleled by 31% decrease in secretion of bile acids while plasma HDL levels and biliary secretion of cholesterol remained unchanged (Hillebrant 1997).

In addition to determining the preferential substrate for secretion into bile, studies have suggested that different species of bile acids may originate from

separate intrahepatic pools of cholesterol (Mitropoulos 1974), delivery into these pools has been suggested to be directed by different species of lipoproteins (Kuipers 1986). This is supported by data suggesting that UC from HDL and chylomicron remnants delivers cholesterol preferentially to the chenodeoxycholic acid pathway, while UC from VLDL preferentially delivers to the cholic acid pathway (Bravo 1990), (Bravo 1992). Together, these studies illustrate the complexities involved in the delivery of substrate to cyp7a and subsequent secretion of bile acids and biliary cholesterol. More work is needed to elucidate the precise mechanisms by which HDL- and/or LDL-derived cholesterol is processed for bile secretion.

1.5.1.3. Regulation of cyp7 enzyme activity by protein phosphorylation

Various *in vitro* studies have suggested the possibility that cyp7a can be regulated by protein phosphorylation (Bjorkhem 1997). Activity of cyp7a isolated from microsomes incubated in the presence of NaF, a protein phosphatase inhibitor, was 80% greater compared to the absence of NaF (Goodwin 1982), (Scallen 1983). Enzyme activity decreased by 40% when microsomes were incubated with alkaline phosphatase (Goodwin 1982). In studies using reconstituted cyp7a, enzyme activity could be completely inhibited upon incubation with alkaline phosphatase and reversed partially upon addition of cyclic-AMP dependent protein kinase A (PKA), suggesting cyp7a enzyme activity is reversibly regulated by phosphorylation/ dephosphorylation (Tang 1986),

(Botham 1992), (Nguyen 1996). The involvement of phosphorylation/dephosphorylation in the regulation of cyp7a remains controversial, as this form of regulation has yet to be described *in vivo*.

1.5.2. Bile secretion

The formation of bile is complex and driven by the secretion of bile acids. The secretion of bile acids by the hepatocyte occurs in an energy-dependent fashion (Nathanson 1991). The hepatocyte possesses a number of ABC transporters specialized in the transport of bile acids, organic ions and neutral amphiphiles against high-concentration gradients into primary bile (Oude Elferink 1999), (Agellon 2000). The bile salt export pump (BSEP; also known as the 'sister of P-glycoprotein' (spgp)) (Childs 1995), (Gerloff 1998) is thought to be the major bile acid exporter in hepatocytes (Gerloff 1998). BSEP has been demonstrated to mediate the ATP-dependent and preferential transport of taurine conjugated bile acids (Gerloff 1998) and mutations in human BSEP are thought to be associated with progressive familial intrahepatic cholestasis (Stautnieks 1998). Another major ABC-transporter involved in bile acid secretion is the multidrug resistance-associated protein 2 (MRP2; also known as canalicular multispecific organic anion transporter (cMOAT)). MRP2/cMOAT has been demonstrated to transport sulfated and glucuronidated bile acids (Kuipers 1989), (Oude Elferink 1989), (Oude Elferink 1995), (Paulusma 1996), (Suzuki 1998). The secreted bile acids promote the solubilization of the canalicular membrane and subsequent extraction of PL, mainly in the form of PC (Meier 2000). The

formation of this mixed micelle promotes the extraction of UC from the canalicular membrane (Meier 2000). The secretion of bile acids and subsequent extraction of UC marks the end of cholesterol catabolism and elimination from the body.

1.6. Specific aims

Recently, our laboratory studied the effect of bile acid flux on the fate of lipoprotein-derived cholesterol in derivatives of the McArdle RH7777 (McA) rat hepatoma cell line (Li 2001). These cells, termed McNtcp. 18 (Torchia 1996), are capable of transporting bile acids and were grown in the presence of HDL or LDL. The presence of lipoproteins in the culture medium reduced *de novo* cholesterol biosynthesis approximately two-fold compared to cells starved in lipoprotein-deficient serum. The rate of cholesterol esterification was 24 times lower in HDL-treated cells despite substantial microsomal ACAT activity compared to LDL-treated cells. Introduction of bile acids to the culture medium reduced both the rate of cholesterol esterification and microsomal ACAT activity by apporximately 50% in LDL-treated cells, but had no effect on enzyme activity in HDL-treated cells. This suggested that HDL-derived cholesterol, unlike LDL-derived cholesterol was not readily accessible for esterification by ACAT. The co-incubation of bile acids in the culture medium of lipoprotein-treated cells resulted in an increase in intracellular UC concentrations. This increase was greater when HDL was the source of cholesterol. The presence of bile acids did not affect uptake of lipoprotein CE or enhance *de novo* cholesterol biosynthesis. However, intracellular CE concentration decreased when cells were treated with bile acids, suggesting that intracellular CE stores were being hydrolyzed. The proportion of lipoprotein-derived UC released into the culture medium was greater in HDL-treated cells compared to LDL-treated cells. This effect was

enhanced increased when lipoprotein-treated cells were co-incubated with bile acids, suggesting the mobilization of cholesterol for biliary secretion. Taken together, these results strongly suggest that HDL- and LDL-derived cholesterol are delivered to different subcellular localizations and are destined for distinct metabolic pools. **The overall goal outlined in this thesis was to investigate the delivery of lipoprotein-derived cholesterol to different subcellular localizations of distinct metabolic relevance in the McArdle RH7777 rat hepatoma cell line.**

Aim 1: To determine the sub-cellular localization of HDL- and LDL-derived cholesterol in McArdle RH7777 rat hepatoma cells

The differences observed in the accessibility of HDL- and LDL-derived cholesterol for esterification and release of lipoprotein-derived cholesterol into the culture medium suggested that HDL- and LDL-derived cholesterol were being delivered to different sub-cellular localizations. Using fluorescence microscopy, the differences in the intracellular distribution of HDL- and LDL-derived cholesterol were examined. Experiments are detailed in Chapter 3.

Aim 2: To determine the metabolic fates of HDL-and LDL-derived cholesterol in McArdle RH7777 rat hepatoma cells

The inaccessibility of HDL-derived cholesterol to ACAT and the enhanced release of HDL-derived UC into the culture medium suggest different metabolic fates for HDL- and LDL-derived cholesterol. Using McA cells stably expressing cyp7a, the processing of HDL- and LDL-derived cholesterol was examined. Experiments are described in Chapter 4.

Chapter 2: Materials and Methods

2.1. Chemicals and laboratory materials

Filipin complex, Mevinolin (commercially known as Lovastatin), cholesteryl oleate standard, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetra acetate (EDTA), diethylenetriaminepentaacetate (DTPA), aminotriazole, bovine serum albumin and 2-hydroxypropyl-β-cyclodextrin was obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Cholesterol standard was purchased from Avanti Polar Lipids (Alabaster, AL). 5-cholesten-3β,7α-diol (hereafter referred to as 7α-hydroxycholesterol) standard was obtained from Steraloids, Inc. (Newport, RI).

Three permanent mammalian cell lines were used in this thesis: the McArdle RH7777 rat hepatoma cell line (McA), McA cells stably expressing cholesterol 7alpha-hydroxylase (hereafter referred to as C15.1) (Labonté 2000), and human skin fibroblasts (HSF EB91-290), a kind gift from Dr. G. Francis (University of Alberta, Edmonton). Cholesterol 7alpha-hydroxylase enzyme activity in C15.1 cells and liver microsomes isolated from a mouse fed a diet containing 2% cholestyramine was measured and shown in Figure II-A (see Appendix II). Cell culture reagents Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), calf serum (CS), phoshphate buffered saline (PBS) and trypsin (0.5% plus 0.53 mM EDTA) were purchased from Invitrogen Canada Inc. (Burlington, ON).

[1α,2α (n)-^{3}H]cholesteryl oleate (specific activity 48.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Inc. (Baie d'Urfe, PQ). [1-^{14}C]

acetate (specific activity 54.3 mCi/mmmol) and [^{14}C]cholesterol (specific activity 55.0 mCi/mmol) were purchased from NEN (Woodbridge, ON). Primary antiserum for SR-BI was purchased from Novus Biologicals, Inc. (Littleton, CO). Primary antiserum for SREBP-2 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary goat-anti-rabbit conjugated horseradish peroxidase (HRP) was purchased from Bio-Rad Laboratories (Cambridge, MA). Primary ABCA1 antiserum was a kind gift provided by Dr. S. Yokoyama (Nagoya City University Medical School, Japan). Human embryonic kidney (HEK) 293 cell homogenates were a kind gift from Dr. G. Francis (University of Alberta, Edmonton). Okadaic acid was a kind gift provided by Dr. C.F.B. Holmes (University of Alberta, Edmonton). Rat liver samples were a kind gift from Dr. R. Lehner (University of Alberta, Edmonton). Rat neurons were a kind gift from Dr. J.E. Vance (University of Alberta, Edmonton). Thin-layer chromatography plates (glass backed Silica Gel G60, 0.25 mm thickness, aluminum backed Silica Gel G60, 0.25 mm thickness) were purchased from BDH Chemicals (Mississauga, ON). Other chemicals and reagents were of analytical grade and purchased from various commercial sources.

2.2. Cell culture

McA cell lines were maintained in DMEM containing 10% FBS and 10% CS at 37°C and 5% CO_2. HSF cells were maintained in DMEM containing 10% FBS at 37°C and 5% CO_2. To passage cells, media was aspirated from

confluent plates. Cells were washed with PBS and overlayed for 30 seconds with 0.5% trypsin. Cells were resuspended in fresh medium and diluted 1/10 into a new culture plate. McA cells were passaged once every three days and HSF cells once a week.

Generally, cells were prepared for experimentation by incubating 70% confluent cultures in DMEM containing 2% bovine LPDS for 16 hours. The cells were then incubated for an additional 16 hours in DMEM containing 2% LPDS plus HDL or LDL at a concentration of 50 μg/ml total cholesterol.

2.2.1. Lovastatin dose-response curve

Cells were prepared for experiment as previously described in Section 2.2. Cells were treated with lipoproteins in the presence of 0-10 μM Lovastatin. *De novo* biosynthesis of cholesterol was measured as described in Section 2.7. and cellular lipids were extracted as described in Section 2.5.3.

2.3. Lipoprotein preparation

2.3.1. Blood collection

Blood, 240 ml was donated from healthy, male volunteers. On ice, each donor's blood was aliquoted in 40 ml fractions into 50 ml Falcon tubes. EDTA was immediately added to a final concentration of 5 mM EDTA. Blood was centrifuged at 3000 rpm at 4°C for 20 minutes in a Beckman GPR tabletop

refrigerated centrifuge (Mississauga, ON) to isolate plasma from blood cells. PMSF (10 µl/100 ml plasma), 100uM DTPA (final concentration) and 1M aminotriazole (1 ml/ 100ml plasma) were added immediately to the isolated plasma, while stirring rapidly for 30 seconds at 4°C.

2.3.2. Lipoprotein isolation and analysis

Plasma lipoproteins were isolated by sequential ultracentrifugation as previously described (Lindgren 1975). The fractions $\rho<1.006$ g/ml (VLDL), $\rho<1.063$ g/ml (LDL) and $\rho<1.021$ g/ml (HDL) were isolated using a Beckman Ti 50.2 rotor (Beckman Coulter, Inc.; Fullerton, CA) centrifuging at 50,000rpm at 8°C for 24 hours.

The identity and migration of each of the lipoproteins were confirmed by agarose gel electrophoresis using the Paragon Lipogel Electrophoresis System for electrophoretic separation of lipoproteins from human plasma, purchased from Beckman Coulter Inc. (Fullerton, CA) (see Figure 3-1). The apolipoprotein composition of HDL and LDL was determined by SDS-PAGE (see Figure 3-2). The oxidation state of purified lipoproteins were tested by measuring thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation and oxidative stress (Armstrong 1994), (Janero 1998). The oxidation state of HDL and LDL were less than 2.5 µM malondialdehyde (MDA) equivalents, an indication of low lipid peroxidation (see Figure 3-3). Total and unesterified cholesterol content of HDL and LDL were determined using commercially

available kits purchased from Wako Chemicals USA, Inc. (Richmond, VA). All lipoproteins were stored under N_2 at 4°C.

2.3.3. Preparation of human lipoprotein-free plasma (LFP) and bovine lipoprotein deficient serum (LPDS)

Human lipoprotein-free plasma (LFP; for radiolabeling of lipoproteins) was collected after removal of HDL following the sequential ultracentrifugation of human plasma. The remaining solution was collected and dialyzed in PBS containing 1 mM EDTA.

Bovine lipoprotein deficient serum (LPDS) was prepared by combining FBS and CS. The density of the solution was measured and adjusted to ρ=1.25 g/ml with solid desiccated KBr. The mixture was then subjected to ultrácentrifugation at 50,000 rpm at 8°C for 24 hours. The lipoproteins were aspirated and discarded. The remaining solution was dialyzed in PBS containing 1 mM EDTA.

2.3.4. Radiolabeling of lipoproteins

Lipoproteins were radiolabeled with [^{3}H] cholesteryl ester as previously described (Roberts 1985). A 250 µCi aliquot of [1α,2α (n)-^{3}H]cholesteryl oleate (Amersham TRK.886) was dried down under a gentle stream of N_2 at room temperature. The dried residues were re-suspended in 500 µl of acetone and this suspension was then added drop-wise to 10 ml of freshly prepared human

lipoprotein-free plasma (LFP) while gently stirring. The acetone was evaporated under a gentle stream of N_2. Human LFP was combined with an equal volume of purified human HDL or LDL (50 mg total cholesterol). The mixture was incubated on a rocker at 4°C for 1 hour. The densities of the mixtures were adjusted to ρ=1.07 g/ml or ρ=1.21 g/ml for LDL or HDL, respectively, with solid desiccated KBr. The radiolabeled lipoproteins were re-isolated as described above. Aliquots of radiolabeled lipoproteins were spotted on TLC plates and developed using the solvent system hexane:diethyl ether:methanol:acetic acid, 70:30:5:1 (v/v). Bands corresponding to unesterified cholesterol and cholesteryl ester standards were visualized by iodine vapor. Bands were scraped and radioactivity determined by liquid scintillation spectrometry.

2.4. Filipin staining and fluorescence microscopy

Cells were stained with filipin using the following protocol (Torchia 1997). Cells were grown on cover-slips in 35 mm plates until 70% confluent. Following treatment, cells were washed thoroughly in PBS and fixed in 4% formalin (pH 7.4) and stained with Filipin stain (250 $\mu g \cdot ml^{-1}$ working concentration), protected from light for 1 hour. Cells were washed in PBS and mounted onto microscope slides with Permafluor (Coulter, Miami, FL) mounting medium. Cells were then viewed under fluorescence microscopy (excitation filters: 338 nm; emission filter: 570 nm) and data were collected using Northern Eclipse image capture software

(Empix Imaging, Inc., Mississauga, ON). Use of the fluorescent microscope kindly provided by Dr. M. Mickalak (University of Alberta, Edmonton).

2.5. Lipid extraction

2.5.1. Modified Folch lipid extraction

Media lipids were extracted by the Folch method (Folch 1957) with the following modifications. Media were collected in 1 ml of aqueous liquid. An equal volume of chloroform:methanol, 2:1 (v/v) was added, vortexed vigorously and incubated at room temperature for 10 minutes. Samples were vortexed a total of five times. One-tenth volume of 50 mM NaCl acidified with 2% acetic acid was added, vortexted and samples left at room temperature for 10 minutes. Samples were centrifuged at 2000 rpm for 30 minutes. The organic phase (bottom) was collected into glass tubes using a glass Pasteur pipette and dried under a gentle stream of N_2. The sides of the tubes were washed with chloroform:methanol to collect lipids at bottom of the tube. Lipids were then re-dried under N_2 and re-suspended in 100-200 µl of chloroform:methanol.

2.5.2. Modified Bligh and Dyer lipid extraction

Cell lipids were extracted by the Bligh and Dyer method (Bligh 1959) with the following modifications. Cells were collected in 1 ml of aqueous liquid into glass tubes. One ml of 100% methanol was added and mixed with the sample.

One ml of acidified NaCl (1M NaCl acidified with 2% acetic acid) was added to the samples. One ml of chloroform was added. While vortexing, methanol was added drop-wise until the solution became monophasic. One ml of acidified NaCl and 2 ml of chloroform was added and samples vortexed. Samples were then subjected to centrifugation at 2000 rpm for 30-60 min to separate organic and aqueous phases. The bottom, organic phase was collected into clean glass tubes. Lipids were dried down under a gentle stream of N_2. Sides of the tube were washed with chloroform:methanol and re-dried under N_2. Samples were re-suspended in 100-200 µl of chloroform:methanol.

2.5.3. Monophasic lipid extraction from cells

To adherent cells on 35 mm dishes, 1 ml of hexane:isopropanol, 3:2 (v/v) was added and incubated at room temperature for 10 minutes. Lipid extract was collected into a glass tube. Plates were washed twice with 1 ml hexane:isopropanol and combined with the first lipid extraction. Lipids were dried under a gentle stream of N_2. Sides of the tube were washed with hexane:isopropanol and re-dried down under N_2. Samples were re-suspended in 100-200 µl of hexane:isopropanol.

2.6. Determination of intracellular cholesterol content

Cells were seeded on 100 mm plates at a density of 2.0×10^6 cells per dish. Following treatment with lipoproteins, cellular lipids were extracted by

method of Bligh and Dyer (described in Section 2.5.2.) and re-suspended in 500 µl hexane. Protein content was determined by method of Bradford (Bradford 1976) using the Bio-Rad Protein Assay commercial diagnostic kit (Bio-Rad Laboratories Inc., Mississauga, ON). Total and unesterified cholesterol content was determined using commercial enzymatic kits. Cholesteryl ester content was calculated by subtracting the unesterified cholesterol content from total cholesterol. All values were normalized to milligrams of cellular protein.

2.7. Measurement of *de novo* cholesterol biosynthesis

Cells were seeded on 35 mm plates at a density of 250,000 cells/dish. Cells were grown overnight in DMEM containing 10% FBS and 10% CS. The cells were washed 3 times in PBS and starved in 2% LPDS for 16 hours. The cells were washed and incubated in the presence of lipoprotein, in DMEM containing 20% serum or DMEM containing 2% LPDS for 16 hours. Cells were then incubated for an additional 4 hours in the presence of 0.3 µCi/ml [1-^{14}C] acetate. The medium was aspirated and cells were washed 4 times in ice cold PBS. Cellular lipids were extracted as described in Section 2.5.3. Following lipid extraction, cellular proteins were extracted by incubating the cells in 0.1 N NaOH for 3 hours at room temperature. Protein content was determined by method of Bradford (Bradford 1976) using the Bio-Rad Protein Assay commercial diagnostic kit (Bio-Rad Laboratories Inc., Mississauga, ON).

Dried lipids were re-suspended in 200 µl hexane:isopropanol and spotted on a thin-layer chromatography plate along with a non-radiolabeled unesterified cholesterol standard. The TLC plate was developed in the solvent system hexane:diethyl ether:methanol:acetic acid, 70:30:5:1 (v/v/v/v). The TLC plate was exposed to a Fuji BAS 1100 Phosphor imaging screen and spots corresponding to the unesterified cholesterol standard were quantitated using the Fuji ImageQuant Software (Fuji Medical Systems, Inc., Stamford, CT). All values are expressed as phosphor-stimulated light (PSL) units as a measure of radioactivity and normalized to milligrams of cellular protein.

2.8. Pulse-Chase

McA or C15.1 cells were grown until 60% confluency in DMEM containing 10% FBS and 10% CS. The cells were washed 3 times in PBS and incubated for 16 hours in DMEM containing 2% LPDS plus non-radiolabeled HDL or LDL at a concentration of 50 µg/ml total cholesterol. Culture medium was removed cells washed 3 times in PBS. Cells were then incubated in DMEM containing 2% LPDS plus radiolabeled HDL or LDL at a concentration of 50 µg/ml total cholesterol for 1 hour. Culture medium was removed and cells were washed 3 times in PBS. The cells were then overlayed with DMEM containing 2% LPDS plus non-radiolabeled HDL or LDL at a concentration of 50 µg/ml total cholesterol and chased for 1-8 hours. Culture medium was collected and cells were washed

4 times with ice cold PBS. Cellular lipids were extracted as described in Section 2.5.2. and medium lipids extracted as described in Section 2.5.1.

In McA cells, lipid residues were re-suspened in 200 ul chloroform: methanol, 2:1 (v/v) and applied to a thin-layer chromatography plate with non-radiolabeled unesterified cholesterol and cholesteryl ester standards. Lipids were separated in the solvent system hexane:diethyl ether:methanol:acetic acid, 70:30:5:1 (v/v/v/v) and bands corresponding to their respective standards were visualized by iodine vapor. Bands were scraped and collected, and radioactivity determined by liquid scintillation spectrometry.

2.8.1. Purification of hydroxylated sterols

In C15.1 cells, following extraction of lipids, aqueous and organic phases were separated into different tubes. Lipids in the organic phase were dried and re-suspended in 200 µl of chloroform:methanol, 2:1 (v/v). Lipids were separated by thin-layer chromatography in the solvent system ethyl acetate:toluene, 60:40 (v/v). The bands corresponding to non-radiolabeled cholesterol, cholesteryl ester and 7α-hydroxycholesterol standards were visualized by iodine vapor. Bands were scraped and collected, and radioactivity determined by liquid scintillation spectrometry. Hydroxylated sterols in the aqueous phase were purified by reversed-phased chromatography using a Sep-Pak cartridge (Waters Corp., Milford, MA). Columns were prepared by sequential washing with 100% and 50% methanol, followed by deionized water. The aqueous phase was applied to the column and eluted with 100% methanol. Eluate was dried-down and re-

suspended in methanol. An aliquot of the purified lipids was quantitated by liquid scintillation spectrometry.

2.9. Microsome preparation

2.9.1. Microsome preparation from cells

Confluent C15.1 cells in 100 mm dishes were washed twice with PBS and once with ice cold microsome homogenization buffer (see Appendix I). On ice, cells from three confluent 100 mm plates were scraped and pooled in 3 ml of microsome homogenization buffer. Cells were sonicated twice at 10 seconds at power 3 in a Ultrasonic Processor Excel sonicator (Heat Systems, Golden, CO). Samples were spun at 9000rpm, 4°C for 20 minutes. Supernatants were then transferred into ultracentrifuge tubes and spun in a TL100.4 rotor at 56000 rpm at 4°C for 1 hour. Supernatant was discarded and the pellet was re-suspended in 250 µl of Cyp7 Assay Buffer (see Appendix I). Protein and unesterified cholesterol content was determined. Microsomes were flash-frozen on dry ice and stored at -70°C.

2.9.2. Microsome preparation from tissues

Liver samples (100-300 mg) were minced with a razor blade and transferred to a Potter-Elvehjem tissue grinder (Wheaton #358003). Tissues were homogenized in ice cold Microsome Homogenization Buffer with 20 strokes

of the Teflon pestle. Homogenates were transferred to Falcon 14 ml plastic snap-cap tubes and spun at 9000rpm at 4°C for 20 minutes. Supernatant was then transferred into ultracentrifuge tubes and spun in a TL100.4 rotor at 56000 rpm at 4°C for 1 hour. Supernatant was discarded and the pellet was then re-suspended in 850 µl of cyp7 assay buffer and aliquoted into 200 µl fractions. Protein and unesterified cholesterol concentrations were determined. Samples were flash-frozen on dry ice and stored at -70°C.

2.10. Cholesterol 7alpha-hydroxylase enzyme activity assay

Cyp7a enzyme activity was measured using a modification of previously described procedures (Martin 1993), (Agellon 1997). Microsomes (100 µg total protein), cyp7 assay buffer and Millipore H_2O were then added to each tube for a final volume of 250 µl. Five µl of [4-^{14}C]cholesterol prepackaged in 2-hydroxypropyl-β-cyclodextrin (10 nCi/nmol) was then added to the iced tube and incubated at 37°C for exactly 3 minutes while shaking. Twenty µl of 10 mM NADPH (freshly prepared in deionized water) and was added to each of the tubes and mixed thoroughly. Tubes were incubated at 37°C for exactly 30 minutes. The reaction was stopped upon addition of 20 µl of 5 N NaOH and tubes were stored on ice. Ethyl acetate was added and tubes were spun briefly to separate aqueous and organic phases. The top (organic) layer was collected into a glass tube and lipids were dried down under a gentle stream of N_2. The walls of the tube were washed with ethyl acetate and re-dried under N_2. Samples

were re-suspended in 100 µl of ethyl acetate and run on a TLC plate and exposed to a Bio-Rad phosporimaging plate overnight. Spots corresponding to the non-radiolabeled 7α-hydroxycholesterol standard was quantitated using Bio-Rad Quantity One™ software (Mississauga, ON).

To determine the incubation time for samples with source cholesterol, a time-course experiment was performed. Figure II-B (see Appendix II) illustrates the conversion of cholesterol to 7α-hydroxycholesterol over time. The results indicate that the enzyme is not saturated at all time-points of the time-course. An incubation time of 30 minutes was chosen for all subsequent experiments.

2.11. Mammalian cell lysis

Liver samples (100-300 mg) were minced with a razor blade and transferred to a Potter-Elvehjem tissue grinder (Wheaton #358003). Tissues were homogenized in 2 ml ice cold PBS with 20 strokes of the Teflon pestle. Adherent cells from a 100 mm dishes were scraped into 5 ml of PBS in a clean plastic tube.

The tissue/cell suspensions were centrifiged at 750 rpm for 20 minutes. Supernatant was aspirated and the pellet re-suspended 100-500 µl mammalian cell lysis buffer. Samples were incubated on ice for 10 minutes. Cell lysates were then centrifuged at 14000 rpm at 4°C for 5 minutes. Supernatant was collected and an aliquot was taken for protein assay. Remaining supernatant stored at -70°C.

2.12. Immunoblot anaysis

All reagents and apparatus for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories Inc. (Mississauga, ON). Following SDS-polyacrylamide gel electrophoresis (Laemmli 1970), proteins were transferred to a PVDF membrane (Millipore, Inc.) by electroblotting (references). Following transfer, the membrane was stained in Ponceau S (see Appendix I) to confirm equal transfer throughout the membrane. The membrane was blocked for 12-16 hours in 5% skim milk powder (w/v) in PBS-Tween (see Appendix I) on a shaker at 4°C. The membrane was incubated for 1 hour with the primary antibody diluted in 5% skim milk powder (w/v) in PBS-T while gently mixing at room temperature. The membrane was washed with PBS-T and incubated for 30 minutes with the secondary antibody conjugated HRP as described above. The membrane was washed in PBS-T and incubated in Enhanced Chemiluminescence (ECL) solution (see Appendix I) for 1 minute. The membrane was then exposed to film.

Primary antiserum of SR-BI was prepared 5% skim milk powder (w/v) in PBS-T at a dilution of 1:5000. Primary antiserum of SREBP-2 was prepared 5% skim milk powder (w/v) in PBS-T at a dilution of 1:1000. Primary antiserum of ABCA1 was prepared in 5% skim milk powder (w/v) in PBS-T at a dilution of 1:1000. Secondary goat anti-rabbit conjugated HRP was prepared in 5% skim milk powder (w/v) in PBS-T at a dilution of 1:10000.

2.13. Statistical analysis

Each figure shown is representative of 3 separate experiments, due to different preparations and differing specific activities of radiolabeled lipoproteins, unless otherwise stated. Values are represented as the mean of triplicate or quadruplicate analyses from one representative experiment.

Chapter 3: Sub-cellular localization of HDL- and LDL-derived cholesterol in McArdle RH7777 cells

3.1. Introduction

As mentioned in Chapter 1, results from our laboratory strongly suggest that LDL-derived cholesterol, but not HDL-derived cholesterol was readily accessible for esterification by ACAT (Li 2001). Also, the introduction of bile acid flux in lipoprotein-treated McNtcp.18 cells resulted in an increase in intracellular UC concentrations, especially when HDL was the source of exogenous cholesterol. Furthermore, the proportion of lipoprotein-derived UC released into the culture medium was greater in HDL-treated cells compared to LDL-treated cells. This effect was enhanced when lipoprotein-treated cells were co-incubated with bile acids, suggesting the mobilization of cholesterol for biliary secretion. The differences in ACAT accessibility and secretion of UC into the culture medium between HDL- and LDL-derived cholesterol suggest differences in their metabolism. It is conceivable that the difference lies in the delivery of lipoprotein-derived cholesterol to different sub-cellular areas within the cell.

In this study, filipin, an antibiotic that binds specifically to UC (Pentchev 1985) (Pentchev 1995) is used as a tool for the fluorescence imaging of UC. It is commonly used in the study of cholesterol storage diseases (Pentchev 1985), (Blanchette-Mackie 2000). Using fluorescence microscopy and various biochemical techniques, the delivery of HDL- and LDL-derived cholesterol to different subcellular localizations is investigated.

3.2. Results

3.2.1. Characterization of purified lipoproteins

Prior to experimental use, lipoprotein preparations were characterized to ensure the quality of the reagents. The identities and migration of each of the purified lipoproteins were confirmed by agarose gel electrophoresis using the Paragon Lipogel Electrophoresis System as described in Section 2.3.2 (Figure 3-1). The apolipoprotein composition of purified HDL and LDL was determined by separating the protein components of the preparations by SDS-PAGE (Figure 3-2). The oxidation state of the purified lipoproteins was determined by measuring thiobarbituric acid reactive substances (TBARS) in each of the preparations as mentioned in Section 2.3.2.. The oxidation states of HDL and LDL was less than 2.5 μM malondialdehyde (MDA) equivalents, an indication of low lipid peroxidation. A sample of oxidized LDL was included in Figure 3-3 as a positive control for oxidized lipoproteins (Figure 3-3). All purified lipoproteins were stored under N_2 at 4°C.

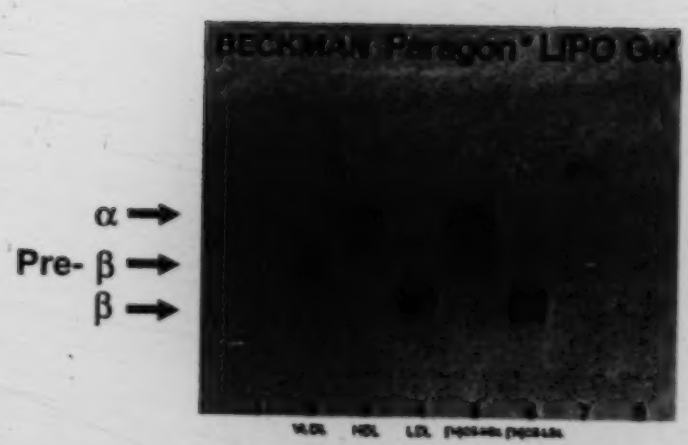


Figure 3-1: Electrophoretic separation of purified human lipoproteins

The identity and migration of each of the lipoproteins were confirmed by agarose gel electrophoresis using the Paragon Lipogel Electrophoresis System (Beckman Coulter Inc., Fullerton, CA). Lipoprotein mobilities (alpha, beta and pre-beta) are shown with arrows to the left of the gel.

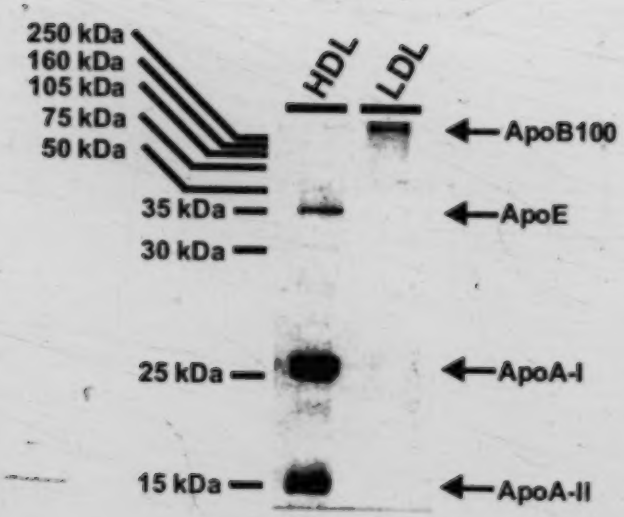


Figure 3-2: Apolipoprotein composition of purified lipoproteins

Apolipoprotein composition of the purified lipoprotein preparations as determined by SDS-PAGE. Mobilities of the apolipoproteins are shown with arrows to the right of the figure.

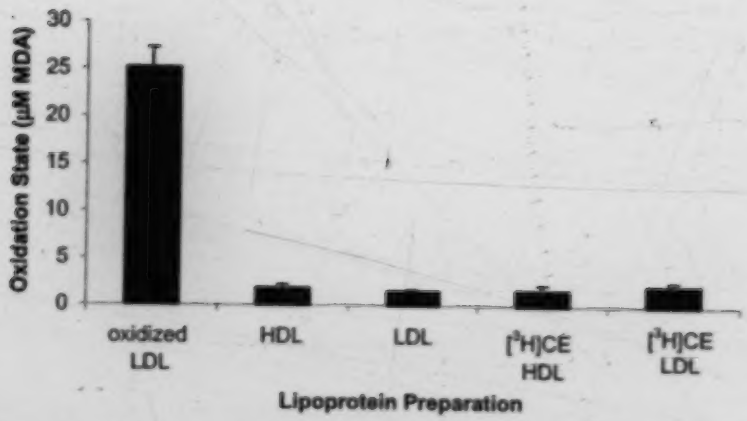


Figure 3-3: Oxidation state of purified lipoproteins

Oxidation state of the purified lipoproteins was determined by measuring thiobarbituric acid reactive substances (TBARS) in each of the preparations. Micromolar malondialdehyde (MDA) equivalents were used as an indicator of TBARS in the lipoprotein preparations.

3.2.2. Intracellular UC distribution by Filipin staining

To determine whether HDL- and LDL-derived cholesterol is targeted to different sub-cellular localizations, human skin fibroblasts (HSF) and McA cells were incubated with purified lipoproteins and stained with filipin. Four sets of cells were treated with complete medium (10% FBS and 10% CS in DMEM), 2% LPDS, HDL and LDL. Figure 3-4 illustrates the UC distribution of lipoprotein-treated cells. HSF cells were used as a control to compare the sub-cellular distribution of UC between a peripheral cell line and a liver-derived cell line. No obvious difference in UC staining was observed between each of the four treatments in HSF cells. In McArdle RH7777 (McA) cells treated with complete medium, 2% LPDS and HDL displayed a punctate distribution of UC throughout the cell with minor peripheral staining. In contrast, cells treated with LDL displayed UC staining localized primarily to the cell periphery. This staining pattern is consistent with biochemical data demonstrating that the majority of LDL-derived cholesterol is destined to the plasma membrane (Neufeld 1998). These results demonstrate that a liver-derived cell line metabolizes cholesterol differently than a peripheral cell line. Futhermore, the results illustrate that HDL- and LDL-derived cholesterol are delivered to different sub-cellular localizations in McA cells.

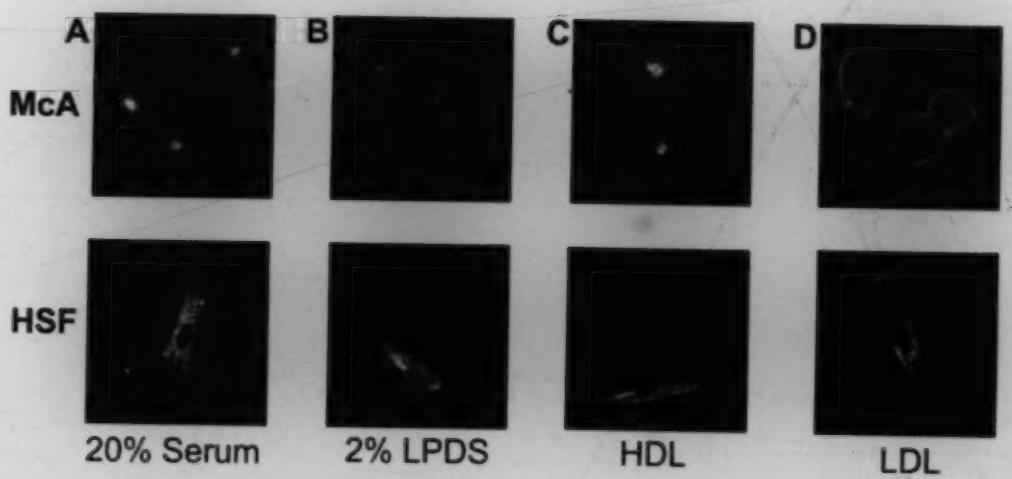


Figure 3-4. Intracellular UC distribution in lipoprotein-treated McA and HSF cells viewed by fluorescence microscopy.

Cells were cultured in medium containing A, complete medium (20% serum; B, 2% LPDS; and 2% LPDS plus purified C, HDL; or D, LDL (50 μg/ml, total cholesterol) for 16 hours, as described. Cells were stained with filipin and visualized by fluorescence microscopy (McA cells: magnification 40X; HSF cells: magnification 20X). Data shown are representative of three different experiments with similar results.

3.2.3. Significance of HDL-mediated cholesterol efflux

HDL-treated cells did not display any obvious accumulation of cholesterol in the plasma membrane. To address the possibility that HDL was responsible for cholesterol removal from McA cells, the ability of HDL- and LDL-derived cholesterol to inhibit *de novo* synthesis of cholesterol was investigated. Previous studies have reported that cells treated with an exogenous source of cholesterol show an inhibition of *de novo* synthesis of cholesterol (Saucier 1989), (Bravo 1993). If HDL caused a net efflux of cholesterol, we would expect *de novo* cholesterol synthesis to be increased. In our experiments, [^{14}C] acetate conversion to cholesterol was employed as an indirect measure of HMGR enzyme activity. Both HDL and LDL reduced [^{14}C] acetate incorporation into cholesterol in comparison to 2% LPDS-treated cells (Figure 3-5). This result demonstrates that HDL is delivering cholesterol to the cells, resulting in an inhibition of *de novo* cholesterol biosynthesis. LDL displayed a greater inhibitory effect on [^{14}C] acetate conversion (3.00-fold greater) compared to HDL.

As mentioned in Chapter 1, ABCA1 is believed to be involved in cholesterol efflux (Oram 2001). The expression of ABCA1 was examined in McA cells (Figure 3-6). HSF was included as a positive control for ABCA1 expression (Bellincampi 2001) and human embryonic kidney (HEK) 293 cells were included as a negative control (Wang 2001), (Tanaka and R. Aoki 2001). Immunoblotting analysis shows an immunoreative band corresponding to ABCA1 in McA cells. There was no apparent change in ABCA1 expressing in

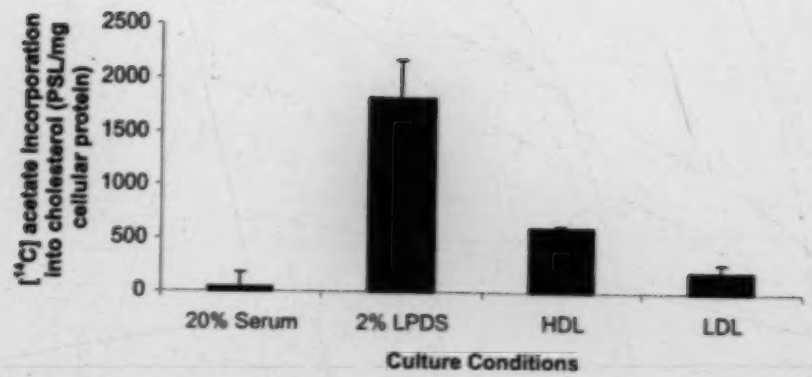


Figure 3-5. Effect of lipoproteins on *de novo* cholesterol biosynthesis. Cells were cultured in medium containing A, complete medium (20% serum, 2% LPDS, and 2% LPDS plus purified HDL or LDL (50 µg/ml, total cholesterol) for 16 hours, as described. Cholesterol biosynthesis was determined by measuring [^{14}C] acetate conversion to cholesterol. Values shown represent mean ± S.D., *n*=3.

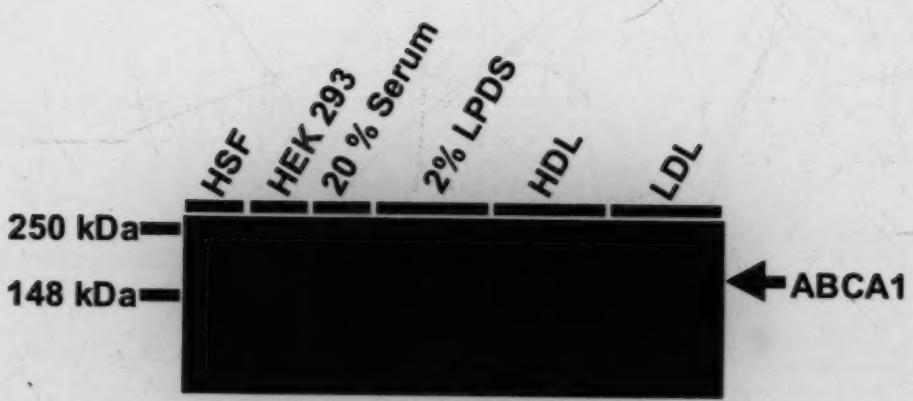


Figure 3-6. Immunoblot analysis of ABCA1 expression in lipoprotein-treated cells.

Preliminary analysis of ABCA1 expression in McA cells. The arrow to the right of the figure indicates the mobility of ABCA1. Each lane was loaded with 30 μg total protein. HSF was included as a positive control, HEK 293 as a negative control. ABCA1 antiserum was used at a dilution of 1:1000.

lipoprotein-treated and starved McA cells.

Because SR-BI is involved in the selective uptake of lipid from HDL, as well as cholesterol efflux (Krieger 1999), SR-BI expression was also examined. As shown in Figure 3-7, immunoblot analysis illustrates that McA cells express SR-BI, suggesting that McA cells are capable of both selective lipid uptake and cholesterol efflux. Expression of SR-BI in rat sympathetic neurons is reported to be undetectable (Posse De Chaves 2000), and was included as a negative control.

To ensure that HDL and LDL was delivering cholesterol to the cells, the mass of UC and CE in lipoprotein-treated cells was determined. In cells cultured in complete medium, or treated with lipoprotein, total intracellular UC content was greater than in cells starved in 2% LPDS (Table I). Intracellular CE contents were similar between each of the treatments with the exception of LDL-treated cells containing significantly greater amounts of intracellular CE. Together, these results suggest that the reduced ability of HDL-derived cholesterol to down-regulate *de novo* cholesterol biosynthesis is not due to a reduction of intracellular cholesterol content.

3.2.4. Unesterified cholesterol distribution in Lovastatin-treated cells

To ascertain whether the UC staining pattern observed in lipoprotein-treated cells is dependent on *de novo* cholesterol synthesis, cells were incubated with HDL or LDL in the presence of 10 μM Lovastatin, a competitive inhibitor of

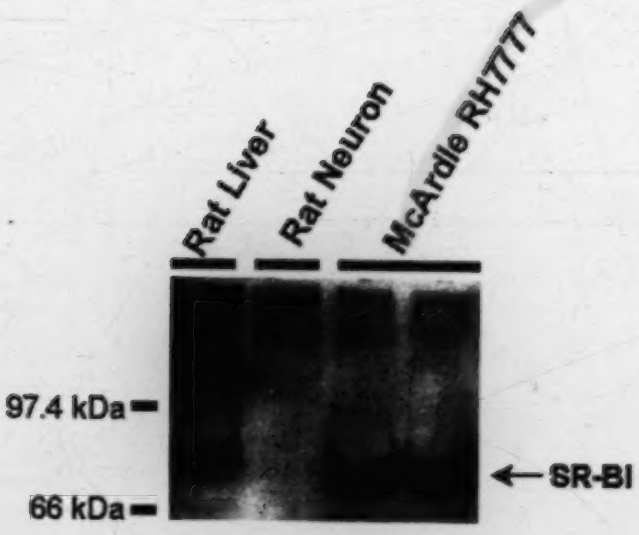


Figure 3-7. Immunoblot analysis of SR-BI expression in McA cells.
The arrow to the right of the figure indicates the mobility of SR-BI. Each lane was loaded with 30 μg total protein. Rat liver was included as a positive control, rat neuron as a negative control. SR-BI antiserum was used at a dilution of 1:5000.

Table I: Total cellular cholesterol content in McA treated with 20% Serum, 2% LPDS, or lipoprotein

Treatment	Unesterified cholesterol (nmol/mg cellular protein)	Cholesteryl Ester (nmol/mg cellular protein)
20% Serum	79.25 ± 7.38*	22.45 ± 7.12
2% LPDS	60.01 ± 17.41	20.77 ± 7.48
HDL	78.03 ± 10.51*	27.38 ± 10.39
LDL	105.33 ± 26.51*	30.59 ± 9.00*

Values shown are ± S.D. and represent data pooled from 3 separate experiments. Differences in comparison to 2% LPDS were evaluated using Student's t-test. *P values of < 0.05 were judged to be significant.

HMGR (McKenney 1988). First, an appropriate inhibitor concentration was determined. As shown in Figure 3-8, [^{14}C] acetate conversion to cholesterol was maximally inhibited at a concentration of 0.1 μM in LDL-treated cells and at 1 μM in HDL-treated cells (Figure 3-8). A concentration of 10 μM was chosen as the working concentration of Lovastatin to maximally inhibit HMGR in subsequent experiments. The UC staining pattern observed in HDL and LDL-treated cells plus Lovastatin was similar to those treated in the absence of Lovastatin (Figure 3-9). These data demonstrate that HDL and LDL-derived cholesterol is delivered to different sub-cellular localizations in McA cells.

3.2.5. Delivery of lipoprotein cholesterol to metabolically distinct pools

The effect on *de novo* cholesterol biosynthesis described above strongly suggests that LDL-derived cholesterol possesses a more potent ability to down-regulate HMGR than HDL-derived cholesterol. Consequently, the ability of LDL-derived cholesterol to further inhibit *de novo* synthesis in HDL-treated cells was explored. Cells treated with 50 μg/ml total HDL-cholesterol were incubated with increasing concentrations of LDL to a total of 50 μg/ml total LDL-cholesterol (Figure 3-10). By increasing the amount of LDL-derived cholesterol delivered to HDL-treated cells, LDL was able to further reduce [^{14}C] acetate conversion to cholesterol. These results strongly suggest that LDL-derived cholesterol has a more potent inhibitory effect on *de novo* cholesterol synthesis and thus, a possible different metabolic fate for HDL- and LDL-derived cholesterol.

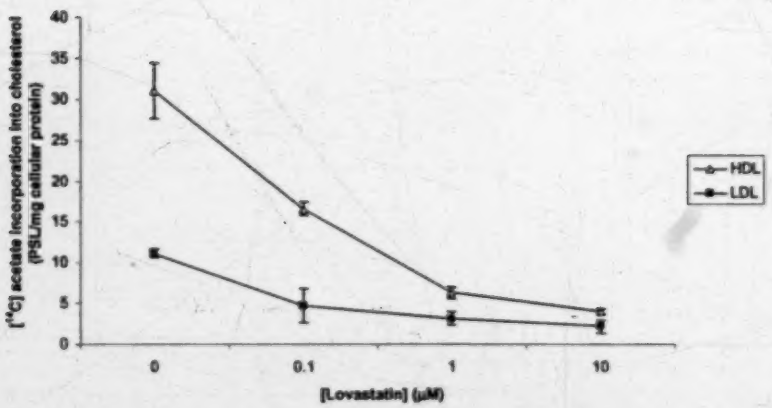


Figure 3-8. Inhibition of *de novo* cholesterol biosynthesis by Lovastatin. Cells were cultured in 2% LPDS plus purified HDL or LDL (50 μg/ml, total cholesterol) in the presence of increasing concentrations of Lovastatin (0.1, 1.0 and 10 μM) for 16 hours. Cholesterol biosynthesis was determined by measuring [^{14}C] acetate conversion to cholesterol. Values shown represent mean ± S.D., *n*=3.

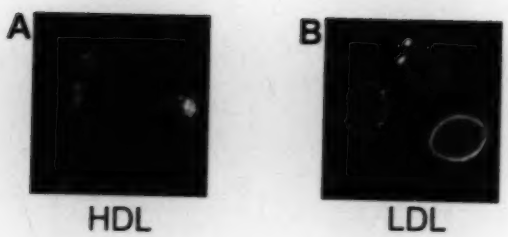


Figure 3-9. Intracellular UC distribution in lipoprotein-treated McA cells in the presence of 10 μM Lovastatin.

Cells were cultured in medium with 2% LPDS plus purified A, HDL; or B, LDL (50 μg/ml, total cholesterol) in the presence of 10 μM Lovastatin for 16 hours, as described. Cells were stained with filipin and visualized by fluorescence microscopy (magnification 40X). Data shown are representative of three different experiments with similar results.

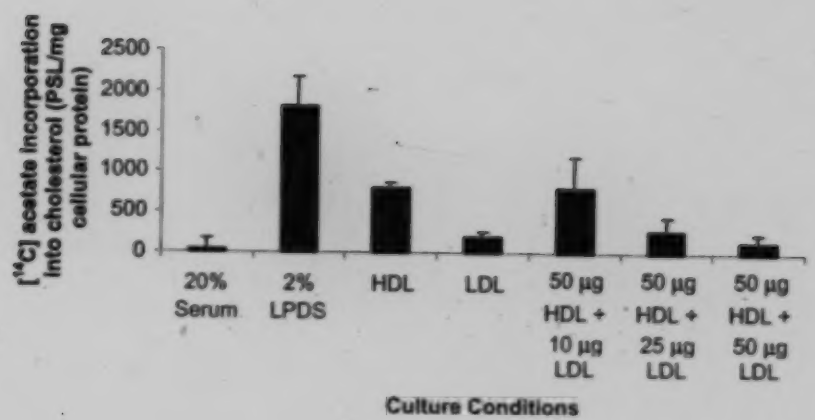


Figure 3-10. Effect of increasing concentrations of LDL in HDL-treated cells on *de novo* cholesterol biosynthesis.

Cells were incubated with purified HDL (50 µg/ml, total cholesterol) were co-incubated with LDL at increasing concentrations (10, 25 and 50 µg/ml, total cholesterol) for 16 hours. Cholesterol biosynthesis was determined by measuring [^{14}C] acetate conversion to cholesterol. Values shown represent mean ± S.D., *n*=3.

As described in Chapter 1, *de novo* cholesterol biosynthesis is controlled at the transcriptional level by SREBPs, predominantly by SREBP-2. In a cholesterol-depleted state, cleavage and release of the 68 kDa subunit of SREBP-2 is up-regulated, resulting in the up-regulation of *de novo* biosynthesis. The difference that was observed in the ability of HDL- and LDL-derived cholesterol to inhibit *de novo* biosynthesis of cholesterol might be explained by a difference in delivery of cholesterol to the site of SREBP-2 regulation. To test this hypothesis, expression of SREBP-2 was measured in cells following treatment with HDL or LDL. Figures 3-11 and 3-12 illustrate preliminary data showing that in starved and HDL-treated cells, the amount of processed 68 kDa SREBP-2 is greater than that of LDL-treated cells. Cells cultured in complete medium show undetectable levels of SREBP-2.

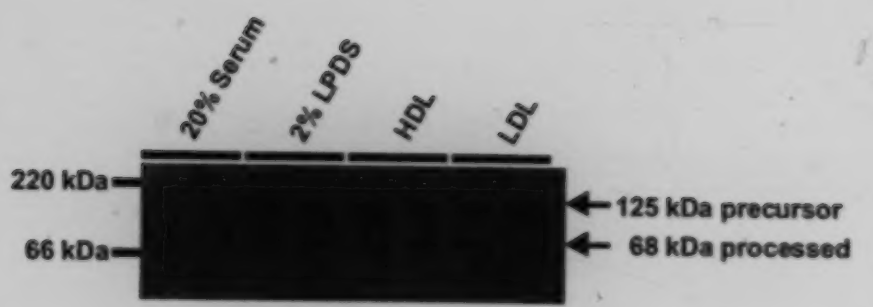


Figure 3-11. Immunoblot analysis of SREBP-2 expression in lipoprotein-treated cells.

Preliminary analysis of SREBP-2 expression and processing. The arrows to the right of the figure indicate the mobilites of the 125 kDa and 68 kDa proteins (precursor and processed, respectively). Each lane was loaded with 30 µg total protein. SREBP-2 antiserum was used at a dilution of 1:1000.

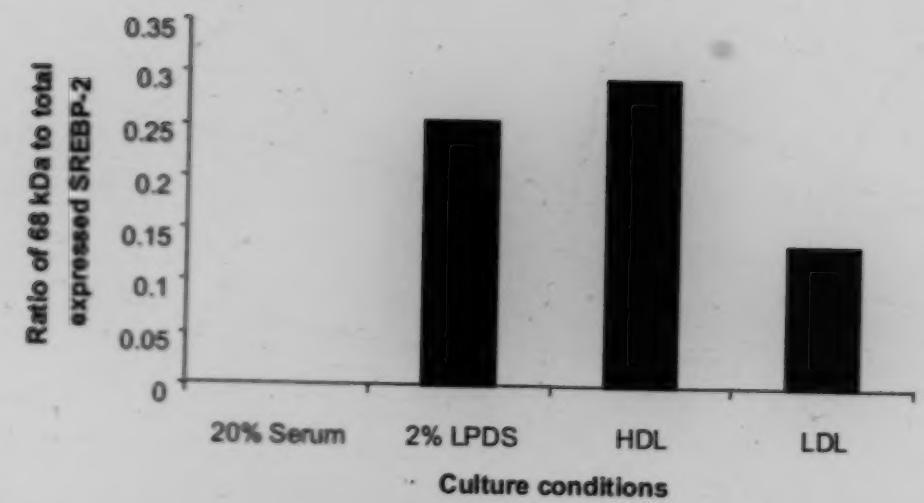


Figure 3-12. Analysis of SREBP-2 processing in lipoprotein-treated cells. The processing of SREBP-2 was examined as the amount of produced 68kDa subunit versus total amount of expressed SREBP-2. Densities were quantitated by densitometry using the using Bio-Rad Quantity One™ software (Mississauga, ON).

3.3. Discussion

Recent data from our laboratory suggest differences in the metabolism of HDL- and LDL-derived cholesterol (Li 2001). In my experiments, investigation of cholesterol distribution in HDL- and LDL-treated cells was examined by filipin staining. In HSF cells treated with lipoproteins, no differences were observed in intracellular distribution of UC. In contrast, in McA cells, there was a difference in the sub-cellular localization of intracellular UC between cells treated with HDL and LDL. These results suggest that peripheral cells metabolize lipoprotein-derived cholesterol differently than a liver-derived cell line and that cholesterol from HDL and LDL are metabolized differently in McA cells.

Consistent with previous reports (Neufeld 1996), UC in LDL-treated cells was localized to the plasma membrane. The UC staining pattern observed in McA cells starved in 2% LPDS is thought to represent newly synthesized cholesterol. HDL-treated cells displayed a similar UC staining pattern to the starved cells. Previous reports have demonstrated that apoA-I from HDL particles possesses the ability to mediate cholesterol efflux from cells (Rothblat 1999), (Oram 1996). It was unclear whether the staining pattern observed was HDL-derived cholesterol, or newly synthesized cholesterol produced as a consequence of the removal of cholesterol by HDL.

It is well documented that there is an inhibition of *de novo* cholesterol biosynthesis upon addition of exogenous sources of cholesterol in cells (Brown 1986), (Bravo 1993). As expected, both HDL- and LDL-derived cholesterol

possessed the ability to inhibit [^{14}C] acetate conversion to cholesterol compared to cells starved in 2% LPDS. LDL-derived cholesterol had a more potent effect on the inhibition of acetate incorporation compared to HDL-derived cholesterol. [^{14}C] Acetate uptake was not measured in HDL- and LDL-treated cells. Differences observed in [^{14}C] acetate incorporation in HDL- and LDL-treated cells may be due to, in part, by differences in uptake.

ABCA1 and SR-BI expression in McA cells was confirmed by immunoblotting. ABCA1 is thought to be involved in cholesterol efflux to apoA-I (Oram 2001) and SR-BI is thought to mediate both selective lipid uptake from HDL, and cholesterol efflux to apoA-I (Krieger 1999). It is possible that the reduced ability of HDL-derived cholesterol to inhibit acetate incorporation was a reduced net delivery of cholesterol caused by cholesterol efflux. It would be expected that depletion of intracellular UC and CE would be indicative of cholesterol efflux. However, measurement of cellular UC and CE mass in HDL-treated cells failed to display such a reduction in intracellular UC and CE, suggesting that in these experiments, apoA-I-mediated cholesterol efflux is not significant.

Lovastatin was used to control for the possible contribution of newly synthesized cholesterol observed in the UC staining of HDL-treated cells. When lipoprotein-treated cells were co-incubated with 10 μM Lovastatin, the UC distribution was virtually identical to cells incubated in the absence of Lovastatin. This suggests that the staining pattern observed in HDL-treated cells in the absence of Lovastatin is unlikely due to HDL-mediated cholesterol efflux.

Furthermore, the UC observed is solely of lipoprotein origin and demonstrates that HDL and LDL-derived cholesterol are delivered to different sub-cellular locations. It is important to note that cells starved in 2% LPDS in the presence of 10 μM Lovastatin were not viable.

The data presented in Figure 3-10 demonstrated that [^{14}C] acetate conversion to cholesterol could be further inhibited in HDL-treated cells upon co-incubation with increasing concentrations of LDL. This result suggested that HDL- and LDL-derived cholesterol might be targeted to different regulatory pools of cholesterol. Preliminary data suggested that in HDL-treated cells, proteolytic cleavage of SREBP-2 was greater than that of LDL-treated cells. The greater amount of transcriptionally active SREBP-2 released in HDL-treated cells could explain the greater amount of [^{14}C] acetate conversion into cholesterol compared to LDL-treated cells. These results further support the hypothesis of HDL- and LDL-derived cholesterol delivery to distinct metabolic pools.

Chapter 4: Processing of lipoprotein-derived cholesterol

4.1. Introduction

The results from Chapter 3 demonstrate that in McA cells, HDL- and LDL-derived cholesterol are delivered to different sub-cellular localizations and differ in their ability to down-regulate HMGR. These results suggest that HDL- and LDL-derived cholesterol have distinct metabolic fates. In this chapter, the processing of lipoprotein-derived cholesterol is further examined. The relative UC:CE ratios in McA cells was measured as an indicator of the amount of lipoprotein-derived cholesterol available for esterification by ACAT.

McA cells have lost the ability to catabolize cholesterol to bile acids. Upon stable expression of cyp7a, the bile acid biosynthetic pathway is reconstituted (Labonté 2000). These cells, referred to as C15.1 cells, are used to investigate the processing of lipoprotein-derived cholesterol. The relative UC:CE ratio was examined in C15.1 cells as an indicator of accessibility of lipoprotein-derived cholesterol to cyp7a for catabolism. Furthermore, the differences observed in accessibility of lipoprotein-derived cholesterol for catabolism led to the investigation of the ability of lipoproteins to modulate the activity of cyp7a.

4.2. Results

4.2.1. Processing of lipoprotein-derived cholesterol in McA cells

To investigate the nature of the processing of HDL- and LDL-derived cholesterol, the targeting of lipoprotein-derived cholesterol was investigated. In these experiments, lipoproteins carried radiolabeled CE, with the label present on the sterol backbone. This allowed for the analysis of lipoprotein-CE processing to intracellular and secreted UC and CE, without interference of endogenous cholesterol pools.

McA cells were pre-conditioned in non-radiolabeled HDL or LDL (50 μg/ml total cholesterol) for 16 hours prior to a 1 hour pulse with [^{3}H] CE-labeled HDL or LDL (50 μg/ml total cholesterol). Cells were then overlayed with medium containing non-radiolabeled HDL or LDL (50 μg/ml total cholesterol) and chased for 1 to 8 hours. In HDL-treated cells, intracellular cholesterol existed predominantly as UC (Figure 4-1A). Levels of intracellular UC decreased over time. This decrease was accompanied by a concomitant increase in UC in the culture medium at the later time-points (Figure 4-1B). Cholesterol secreted into the medium existed predominantly in the form of UC. Intra- and extra-cellular CE levels remained relatively unchanged over time. In LDL-treated cells, intracellular cholesterol also existed predominantly as UC (Figure 4-2A). Intracellular levels of UC and CE remained relatively unchanged over time. In

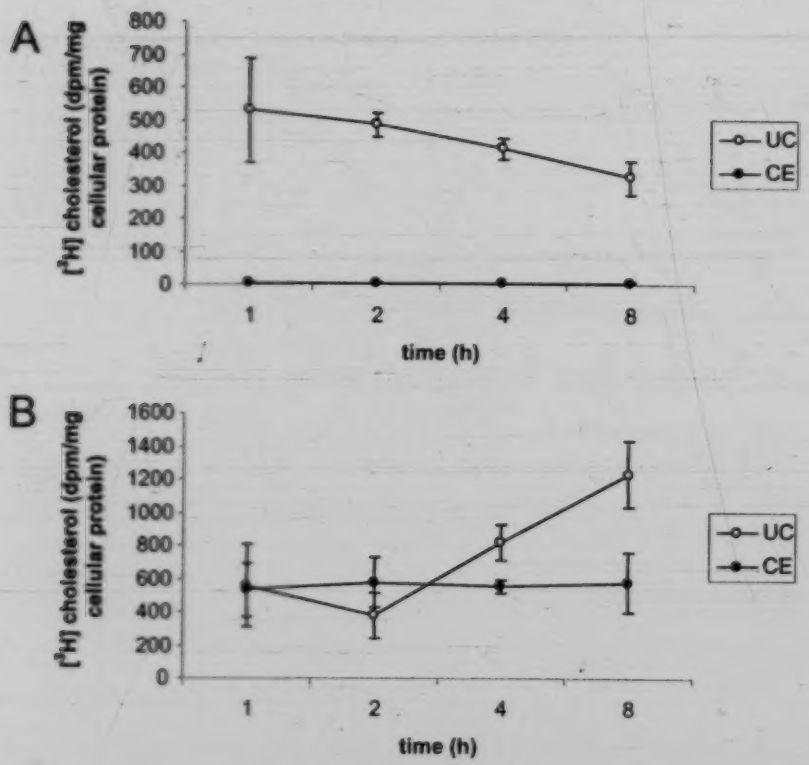


Figure 4-1. Processing of HDL-derived cholesterol to UC and CE in McA cells.

Cells pre-conditioned in medium containing non-radiolabeled HDL were pulsed for 1 hour with radiolabeled [^{3}H]CE-HDL, and chased with medium containing non-radiolabeled HDL (lipoprotein concentration 50 μg/ml). A, the processing of HDL-derived [^{3}H]CE to intracellular UC and CE. B, the secretion of radiolabeled UC and CE into the culture medium derived from [^{3}H]CE-HDL. Values shown represent mean ± S.D., *n*=4.

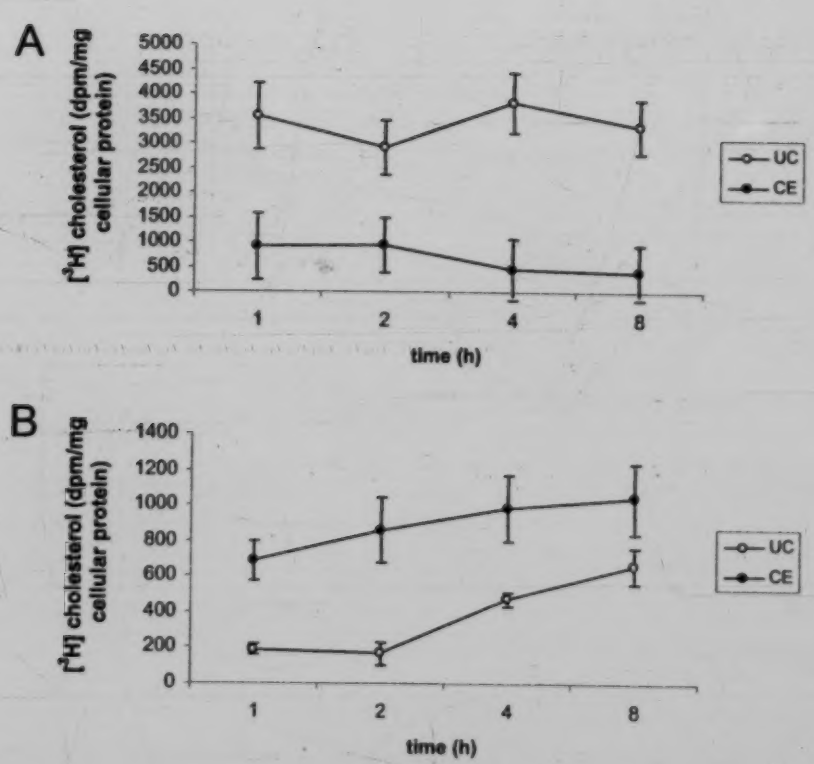


Figure 4-2. Processing of LDL-derived cholesterol to UC and CE in McA cells.

Cells pre-conditioned in medium containing non-radiolabeled LDL were pulsed for 1 hour with radiolabeled [^{3}H]CE-LDL, and chased with medium containing non-radiolabeled LDL (lipoprotein concentration 50 μg/ml). A, the processing of LDL-derived [^{3}H]CE to intracellular UC and CE. B, the secretion of radiolabeled UC and CE into the culture medium derived from [^{3}H]CE-LDL. Values shown represent mean ± S.D., *n*=4.

contrast to HDL-treated cells, secreted cholesterol in LDL-treated cells existed predominantly as CE (Figure 4-2B). Using the data above, the intracellular and secreted UC:CE ratios were calculated. The UC:CE ratio was greater in HDL-treated cells compared to LDL-treated cells (Figure 4-3). This is observed both intracellularly (at 8 hours, UC:CE ratio is 54.9 and 9.1 for HDL and LDL, respectively), and extracellularly (at 8 hours, UC:CE ratio is 2.2 and 0.6 for HDL and LDL, respectively). Consistent with data provided by Li, *et al.*, radiolabeled HDL- and LDL-CE was targeted to different intracellular pools with different accessibility to ACAT (Li 2001).

4.2.4. Processing of lipoprotein cholesterol in C15.1 cells

As previously mentioned, McA cells have lost the ability to catabolize cholesterol to bile acids. This may account for the increased UC:CE ratio observed in HDL-treated cells. Thus, the accessibility of HDL- and LDL-derived cholesterol to cyp7a was examined in C15.1 cells, in which the bile acid biosynthetic pathway has been reconstituted (Labonté 2000).

The intracellular cholesterol targeting patterns were similar in C15.1 cells to that of McA cells. In HDL-treated cells, the majority of intracellular lipoprotein-derived cholesterol was in the form of UC (Figure 4-4A). There was a decrease in intracellular UC over time, paralleled by a concomitant increase in UC secretion into the culture medium (Figure 4-4B). Intra- and extracellular CE levels remained relatively unchanged. In LDL-treated cells, intracellular

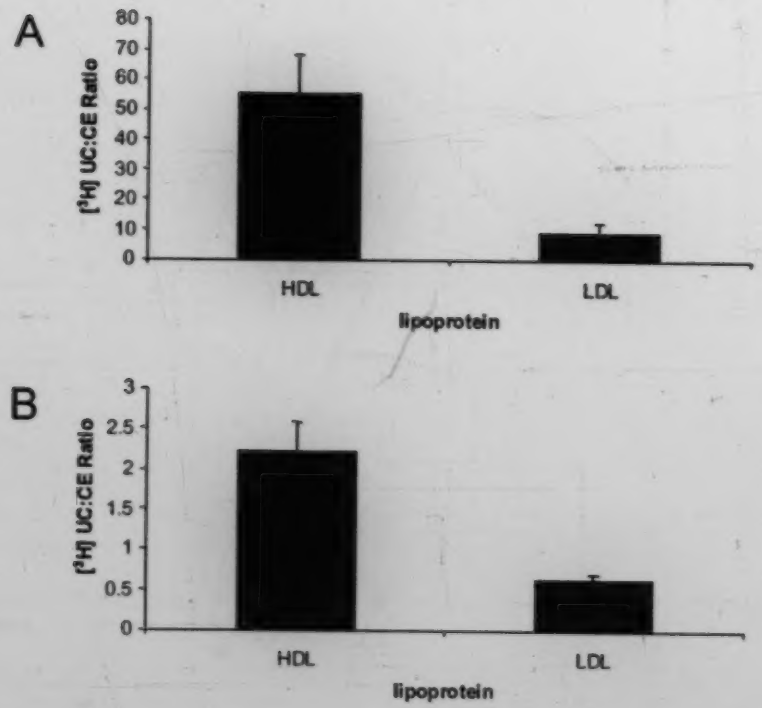


Figure 4-3. Calculation of [^{3}H] UC:CE Ratio

A, Intracellular; and B, Secreted [^{3}H] labeled UC:CE ratios were calculated from the values shown in Figures 4-4 and 4-5 above at the 8 hour time-point. Values shown represent mean ± S.D., *n*=4.

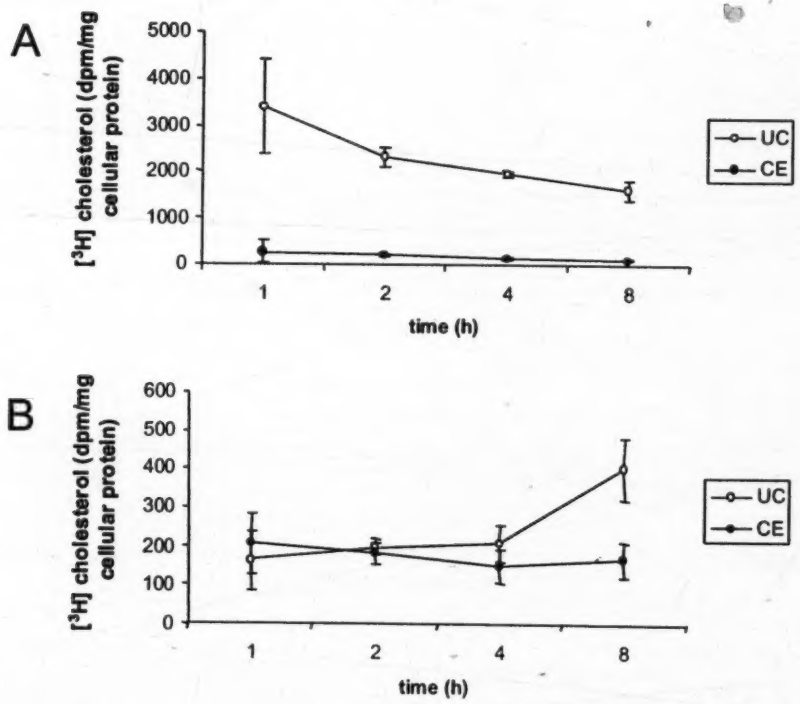


Figure 4-4. Processing of HDL-derived cholesterol to UC and CE in C15.1 cells.

Cells pre-conditioned in medium containing non-radiolabeled HDL were pulsed for 1 hour with radiolabeled [^{3}H]CE-HDL, and chased with medium containing non-radiolabeled HDL (lipoprotein concentration 50 μg/ml). A, the processing of HDL-derived [^{3}H]CE to intracellular UC and CE. B, the secretion of radiolabeled UC and CE into the culture medium derived from [^{3}H]CE-HDL. Values shown represent mean ± S.D., *n*=4.

lipoprotein-derived cholesterol was predominantly in the form of UC (Figure 4-5A). In the culture medium, the secretion of cholesterol existed predominantly as CE (Figure 4-5B).

Like McA cells, the UC:CE ratio in HDL-treated C15.1 cells was greater than that of LDL-treated cells both intra- and extra-cellularly (Figure 4-6). Comparing of the UC:CE ratio in C15.1 cells to McA cells, the introduction of cyp7a resulted in a sharp decrease in the intracellular UC:CE ratio in HDL-treated cells (3.87-fold reduction) whereas the intracellular UC:CE ratio in LDL-treated cells remained relatively unchanged. The extracellular UC:CE ratio in HDL-treated cells remained unchanged, whereas there was an observed decrease in extracellular UC:CE ratio in LDL-treated cells (1.78-fold reduction). The reduction in UC:CE ratio strongly suggests that upon the expression of cyp7a, a pathway is now available for which HDL-derived cholesterol to be catabolized.

To control for the contribution of newly synthesized cholesterol, C15.1 cells were treated with lipoproteins in the presence of 10 μM Lovastatin (Figure 4-7). Preliminary data showed that when *de novo* cholesterol synthesis was inhibited, the intracellular UC:CE ratio further increased in HDL-treated cells (1.56-fold increase), while extracellular UC:CE ratio remained unchanged. In contrast, in LDL-treated cells, the intracellular UC:CE ratio decreased further (3.35-fold reduction), while the extracellular UC:CE ratio was unchanged.

As shown above, the expression of cyp7a affects the UC:CE ratio in HDL-treated cells. This implies that HDL-derived cholesterol may be more accessible

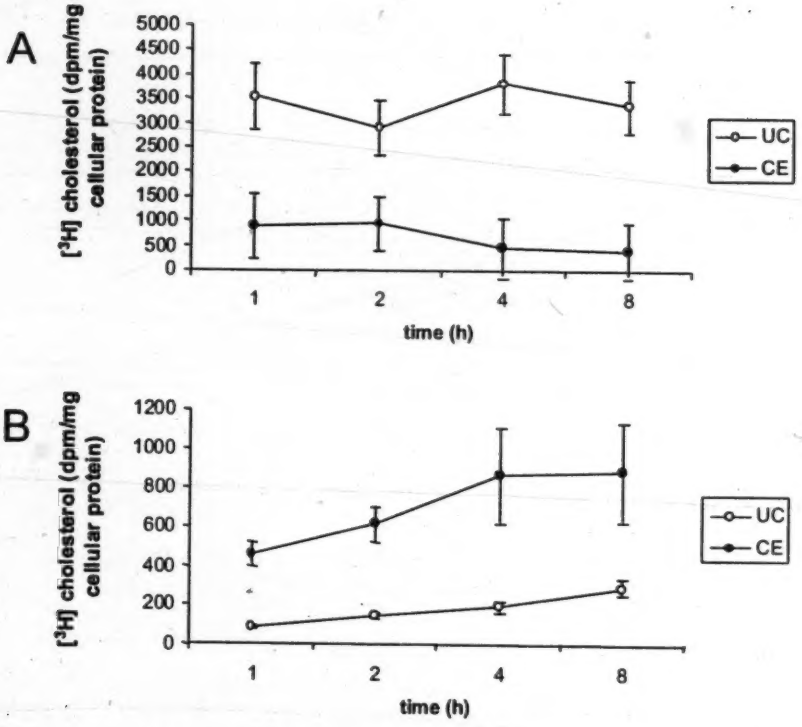


Figure 4-5. Processing of LDL-derived cholesterol to UC and CE in C15.1 cells.

Cells pre-conditioned in medium containing non-radiolabeled LDL were pulsed for 1 hour with radiolabeled [^{3}H]CE-LDL, and chased with medium containing non-radiolabeled LDL (lipoprotein concentration 50 µg/ml). A, the processing of LDL-derived [^{3}H]CE to intracellular UC and CE. B, the secretion of radiolabeled UC and CE into the culture medium derived from [^{3}H]CE-LDL. Values shown represent mean ± S.D., *n*=4.

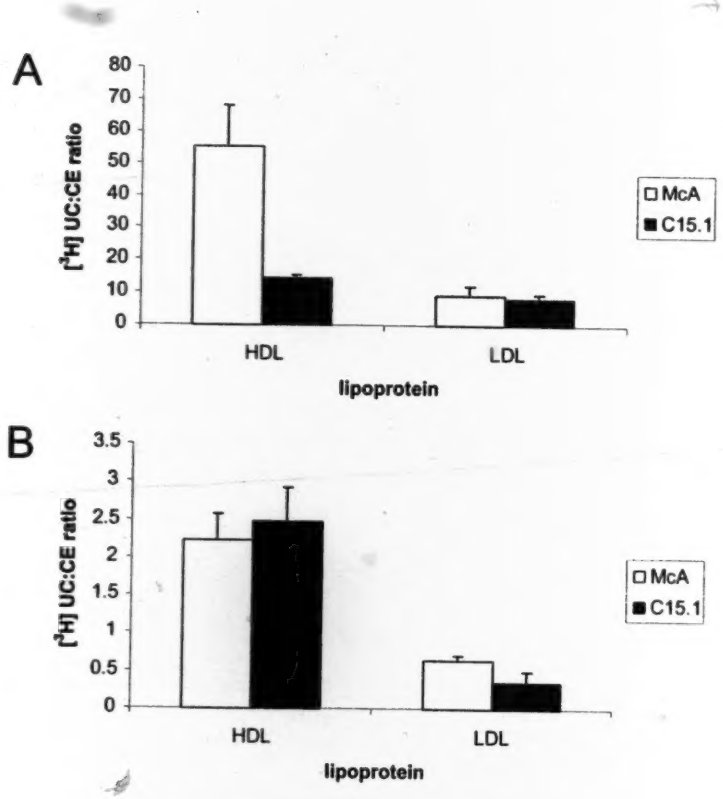


Figure 4-6. Comparison of [^{3}H] UC:CE ratio between McA and C15.1 cells. A, Intracellular, and B, secreted [^{3}H] labeled UC:CE ratios were calculated from the data above at the 8 hour time-point. McA cells are depicted as open bars; C15.1 cells as closed bars. Values shown represent mean ± S.D., *n*=4.

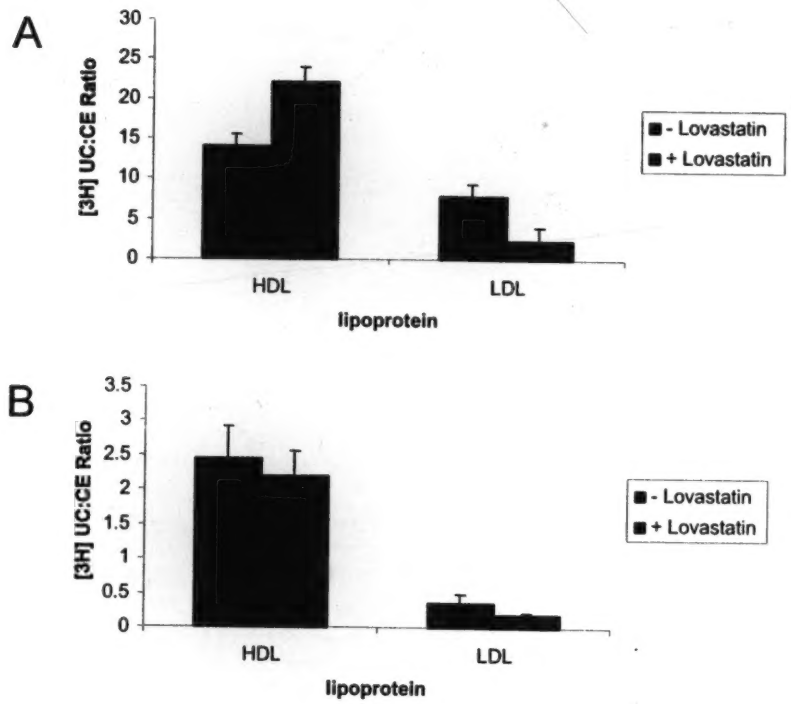


Figure 4-7. Effect of Lovastatin on [^{3}H] UC:CE ratio in lipoprotein-treated C15.1 cells.

Preliminary data illustrating calculated A, intracellular; or B, secreted [^{3}H] labeled UC:CE Ratio at 8 hours. Cells treated in the absence of Lovastatin are depicted as dark bars; in the presence of Lovastatin as shaded bars. Values shown represent mean ± S.D., *n*=4.

to cyp7a for catabolism. To test this hypothesis, the catabolism of lipoprotein-derived cholesterol was examined. In these experiments, the product of cholesterol catabolism is expressed as total produced hydroxylated sterols. Total production of hydroxylated sterols over the 8-hour chase is depicted in Figure 4-8. In HDL-treated cells, levels of intracellular hydroxylated sterols decreased over time. This decrease was accompanied by a concomitant increase in hydroxylated sterol in the culture medium. In contrast, in LDL-treated cells intracellular and secreted hydroxylated sterol levels remained relatively unchanged. After normalizing to lipoprotein specific activity, a greater production of hydroxylated sterols is detected when HDL is the source of cholesterol compared to that of LDL.

To account for the utilization of newly synthesized cholesterol by cyp7a, the production of hydroxylated sterols was investigated in cells treated with lipoproteins in the presence of 10 μM Lovastatin. The results illustrated in Figure 4-9, show that the levels of produced hydroxylated sterols remained relatively unchanged in cells treated with Lovastatin compared to cells treated without Lovastatin.

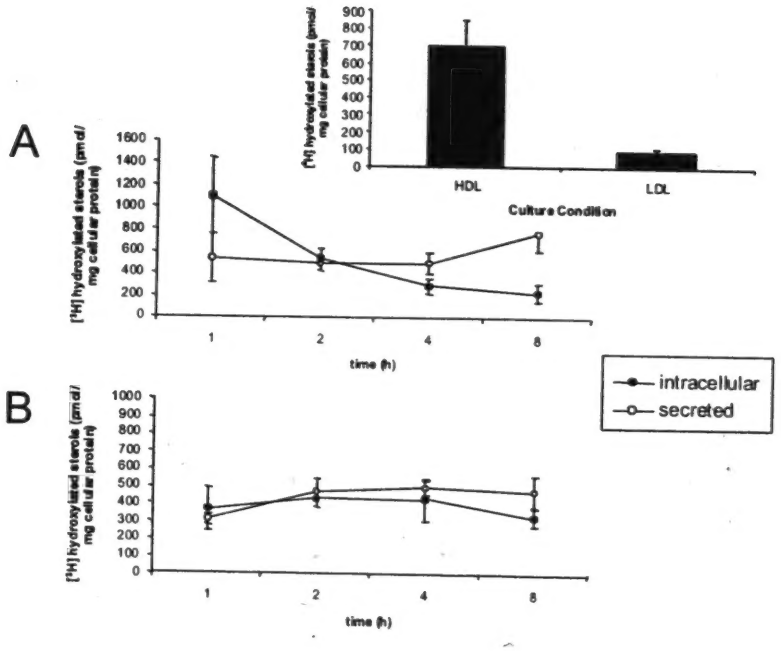


Figure 4-8. Production of hydroxylated sterols in HDL- and LDL-treated C15.1 cells.

A, processing of HDL-derived [^{3}H]CE to intracellular (black squares) and secreted (open circles) hydroxylated sterols. B, processing of LDL-derived [^{3}H]CE to intracellular (black squares) and secreted (open circles) hydroxylated sterols. Inset, total production of [^{3}H] hydroxylated sterols at 8 hours. Normalized to lipoprotein specific activity. Values shown represent mean ± S.D., *n*=4.

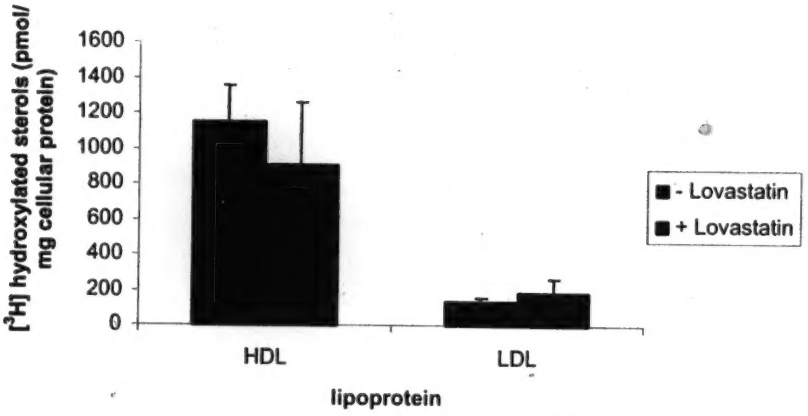


Figure 4-9. Effect of Lovastatin on hydroxylated sterols production C15.1 cells

Hydroxylated sterol production in C15.1 cells treated with lipoproteins in the presence (shaded bars) or absence (dark bars) of Lovastatin, at 8 hours. Values shown represent quadruplicate analyses from a single experiment, mean ± S.D.

4.2.3. Effect of lipoproteins on cyp7a enzyme activity in C15.1 cells

The difference observed in the conversion of lipoprotein-derived cholesterol to hydroxlated sterols suggests potential post-translational regulation of cyp7a enzyme activity mediated by HDL and LDL. Consequently, the activity of cyp7a was measured following treatment of C15.1 cells with lipoproteins. As illustrated in Figure 4-10, treatment with lipoproteins had no detectable effect on the modulation of cyp7a enzyme activity. This result suggests that the difference observed in the accessibility of lipoprotein-derived cholesterol is not due to post-translational modulation of cyp7a enzyme activity.

4.2.4. Effect of hyper-phosphorylation on cyp7a enzyme activity

As mentioned in Section 1.5.1.3., *in vitro* studies suggest that cyp7a enzyme activity is up-regulated in its phosphorylated state. Protein kinase A has been implicated as being responsible for this regulation (Goodwin 1982), (Tang 1986), (Nguyen 1996). Okadaic acid (OA), an inhibitor of protein phosphatase (PP)-1 and 2A (Hardie 1991), was used as a tool to induce C15.1 cells to a hyper-phosphorylated state. Following induction of hyper-phosphorylation, cyp7a enzyme activity was measured. As shown in Figure 4-11, incubation of cells with OA caused a decrease in cyp7a enzyme activity.

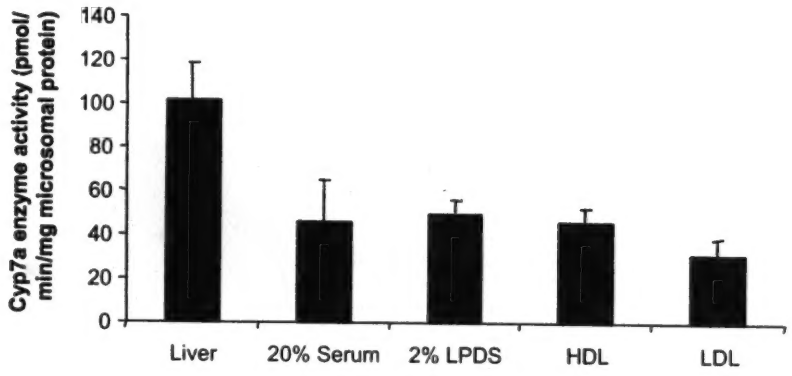


Figure 4-10. Effect of lipoproteins on the modulation of cyp7a enzyme activity.

C15.1 cells were treated for 16 h in medium containing 2% LPDS plus HDL or LDL (50 μg/ml, total cholesterol). Cellular cyp7a enzyme activity was compared to cholestyramine-fed mouse liver. Microsomes were prepared as described and cyp7a enzyme activity determined. Values shown represent ± S.D., *n*=3.

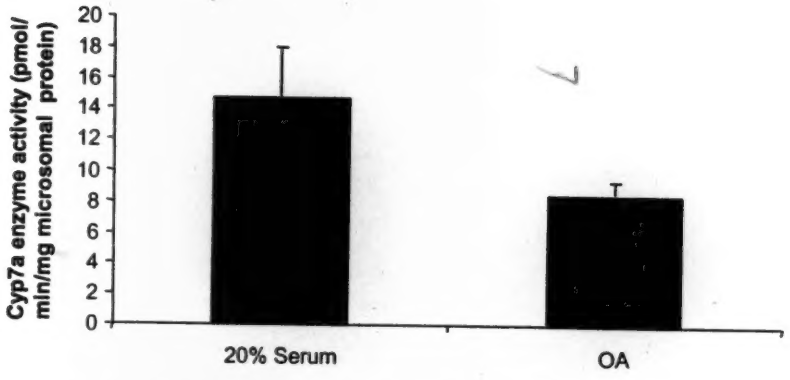


Figure 4-11. Effect of okadaic acid on cyp7a enzyme activity.

C15.1 cells cultured in 20% Serum were incubated for 30 min in meduim containing 20% serum and 1 μM okadaic acid (OA). Microsomes were prepared as described and cyp7a enzyme activity determined. Values shown represent ± S.D., *n*=3.

4.3. Discussion

As mentioned in Section 4.1., the calculated UC:CE ratio was used as an indicator for the accessibility of lipoprotein-derived cholesterol for esterification by ACAT. Consistent with previous data from our laboratory (Li 2001), the low UC:CE ratio observed in LDL-treated cells represents an ACAT accessible pool of cholesterol, whereas, the high UC:CE ratio observed in HDL-treated cells is not.

In C15.1 cells, the UC:CE ratio in HDL-treated cells was significantly reduced, whereas the UC:CE ratio in LDL-treated cells remained relatively unchanged when compared to McA cells. In McA cells, the ability to catabolize cholesterol is lost. Stable expression of cyp7a however, restores the bile acid biosynthetic pathway (Labonté 2000) providing a pathway for which cholesterol can be catabolized. The build-up of UC in HDL-treated McA cells is suggestive of the cells' lost ability to catabolize bile acids. This build-up is released following the expression of cyp7a. The production of hydroxylated sterols from lipoprotein-derived cholesterol was determined as a measure of substrate accessibility to cyp7a. As expected, total production of lipoprotein-derived cholesterol to hydroxylated sterols was greater in HDL-treated cells compared to LDL-treated cells. Furthermore, the excess UC existing in, and secreted by HDL-treated C15.1 cells, in addition to being more accessible for bile acid production, may also represent cholesterol destined for biliary secretion. These results strongly support previous reports that HDL-derived cholesterol is the preferential source

of cholesterol for bile acids (Bravo 1989) and biliary cholesterol (Robins 1997), (Kozarsky 1997), (Ji 1999). In contrast, the observed predominance of CE production and secretion in both McA and C15.1 cells treated with LDL suggest that LDL-derived cholesterol might instead, be destined for esterification by ACAT and subsequently, for lipoprotein secretion.

To control for dilution of radiolabel by newly synthesized cholesterol, *de novo* cholesterol biosynthesis was inhibited by co-incubation with Lovastatin. Interestingly, the inhibition of *de novo* cholesterol synthesis did not produce an enhancement in hydroxylated sterol production in both HDL- and LDL-treated cells, suggesting that in C15.1 cells, the utilization of newly synthesized cholesterol to cyp7a is minimal in the presence of lipoproteins. Intracellular UC:CE ratio increased further in HDL-treated C15.1 cells, whereas intracellular UC:CE ratio decreased further in LDL-treated cells. These results demonstrate that targeting of HDL- and LDL-derived cholesterol remain unchanged, with or without the contribution of newly synthesized cholesterol. These results reconfirm the hypothesis that HDL- and LDL-derived cholesterol is destined for distinct metabolic pools.

The differences observed in the conversion of HDL- and LDL-derived cholesterol to hydroxylated sterols led to the investigation of the ability of lipoproteins to modulate cyp7a enzyme activity post-translationally. Treatment of C15.1 cells with HDL or LDL did not alter cyp7a enzyme activity, suggesting that lipoproteins do not exert post-translational regulation of cyp7a. Previous *in vitro* studies have suggested that phosphorylation of cyp7a results in an up-regulation

of its activity (Goodwin 1982), (Tang 1986) (Nguyen 1996). Interestingly, forcing hyper-phosphorylation of cyp7a by okadaic acid resulted in a down-regulation of enzyme activity. This result suggests that cyp7a is modulated by protein phosphorylation, but is down-regulated in its phosphorylated state. Taken together, the results strongly suggest that the differences observed in conversion of lipoprotein-derived cholesterol to hydroxylated sterols is due to substrate availability to cyp7a and not to lipoprotein-mediated post-translational modulation of enzyme activity.

Chapter 5: Summary and Conclusion

5.1. Summary

The liver plays a pivotal role in the regulation of total body cholesterol homeostasis. The metabolism of HDL- and LDL-derived cholesterol by the liver is central to this process. Perturbation of these homeostatic mechanisms can disrupt a delicate balance, leading to disease such as atherosclerosis.

It has been suggested that LDL is responsible for the delivery of cholesterol to the peripheral tissues, and that HDL is responsible for the return of excess cholesterol back to the liver for elimination in bile. This hypothesis is supported by clinical evidence when elevated plasma LDL levels increase the risk of atherosclerosis development (Lusis 2000). Conversely, there is an inverse correlation between plasma HDL-levels and atherosclerosis development (Gordon 1989). This led to the hypothesis of the RCT pathway, whereby HDL mediates the removal of cholesterol, thus protecting against the accumulation of cholesterol in the periphery as a result of LDL (Glomset 1968).

One of the major differences in the atherogenic potential of HDL and LDL is hypothesized to lie in their metabolism by the liver. HDL- and LDL-derived cholesterol has been shown to be internalized by different mechanisms. Further, following the internalization of this cholesterol, differences in its intracellular trafficking have been reported (Schroeder 2001). It is therefore conceivable that the differences observed in the functions of HDL and LDL reflects differences in their intracellular targets.

Recently, our laboratory studied the effect of bile acid flux on the fate of lipoprotein-derived cholesterol in McArdle RH7777 rat hepatoma cells capable of transporting bile acids (Li 2001). These cells, termed McNtcp. 18 (Torchia 1996), were grown in the presence of HDL or LDL. The rate of cholesterol esterification was 24 times lower in HDL-treated cells compared to LDL-treated cells despite substantial microsomal ACAT activity in HDL-treated cells. Introduction of bile acids to the culture medium reduced both the rate of cholesterol esterification and microsomal ACAT activity by apporximately 50% in LDL-treated cells, but had no effect on enzyme activity in HDL-treated cells. These suggest that HDL-derived cholesterol, unlike LDL-derived cholesterol was not readily accessible for esterification by ACAT. The co-incubation of bile acids in the culture medium of lipoprotein-treated cells resulted in an increase in intracellular UC concentrations. This increase was greater when HDL was the source of cholesterol. The presence of bile acids did not enhance lipoprotein CE uptake nor *de novo* cholesterol biosynthesis. However, intracellular CE concentration decreased when cells were treated with bile acids, suggesting that intracellular CE stores were being hydrolyzed. The proportion of lipoprotein-derived UC released into the culture medium was greater in HDL-treated cells compared to LDL-treated cells. This effect was enhanced when lipoprotein-treated cells were co-incubated with bile acids, suggesting the mobilization of cholesterol for biliary secretion. Taken together, these results strongly suggest that HDL- and LDL-derived cholesterol are delivered to different subcellular localizations and are destined for distinct metabolic pools.

The differences observed in the aforementioned study directed us to investigate the sub-cellular distribution of HDL- and LDL-derived cholesterol. The first objective of the thesis was to determine whether HDL- and LDL-derived cholesterol was delivered to different sub-cellular localizations. The results from Chapter 3 illustrate a difference in the distribution of HDL- and LDL-derived cholesterol by fluorescence imaging of UC in McA cells. The delivery to these distinct intracellular localizations strongly suggested that HDL- and LDL-derived cholesterol are delivered to distinct metabolic pools.

HDL- and LDL-derived cholesterol was shown to have a different effect on their ability to down-regulate *de novo* cholesterol biosynthesis. This was correlated by preliminary evidence that HDL- and LDL-treated cells differed in the processing of SREBP-2. These results further suggest that HDL- and LDL-derived cholesterol differ in their metabolic fates.

The processing of lipoprotein-derived cholesterol in McA and C15.1 cells was investigated in Chapter 4. The difference in sub-cellular UC distribution and ability to down-regulated *de novo* cholesterol biosynthesis strongly suggested a difference in the processing and metabolic targeting of lipoprotein-derived cholesterol. Accessibility of lipoproprotein-derived cholesterol to ACAT was investigated by measuring the UC:CE ratios in HDL- and LDL-treated McA cells. HDL-treated cells displayed a high UC:CE ratio indicative of a build-up of intracellular UC. In contrast, LDL-treated cells had a much lower UC:CE ratio, due to a greater amount of intracellular CE. Furthermore, the secretion of cholesterol in HDL-treated cells was in the form of UC, whereas in LDL-treated

cells, it was in the form of CE. This supports previous studies suggesting the LDL- but not HDL-derived cholesterol was accessible for esterification by ACAT (Li 2001).

Upon expression of cyp7a, the build-up of intracellular UC in HDL-treated cells was markedly reduced, whereas in LDL-treated cells, it remained relatively unchanged. It was expected that cyp7a would utilize cholesterol from the HDL-derived pool. This was supported by the observation that HDL-treated cells produced more hydroxylated sterols than LDL-treated cells. In addition, cholesterol secreted into the culture medium in HDL-treated cells was in the form of UC. These results support previous studies that suggest that HDL is the preferred source of cholesterol for bile acids (Bravo 1993), (Bravo 1994) and biliary cholesterol secretion (Schwartz 1978), (Bravo 1989), (Kozarsky 1997), (Robins 1997), (Wang 1998), (Sehayek 1998), (Ji 1999). As in McA cells, cholesterol secreted into the medium in LDL-treated cells was mainly in the form of CE.

Lovastatin was used to control for dilution of radiolabel by newly synthesized cholesterol. Interestingly, inhibition of *de novo* cholesterol synthesis did not result in an enhancement of hydroxylated sterol production in both HDL- and LDL-treated cells. This suggested that in C15.1 cells, the utilization of newly synthesized cholesterol by cyp7a is minimal in the presence of lipoproteins. In HDL-treated C15.1 cells, the intracellular UC:CE ratio increased while in LDL-treated C15.1 cells, the intracellular UC:CE ratio decreased. The results demonstrate that the utilization of HDL- and LDL-derived cholesterol by cyp7a

remains unchanged, with or without the contribution of newly synthesized cholesterol.

The difference observed in the conversion of HDL- and LDL-derived cholesterol to hydroxylated sterols suggested the possibility that lipoproteins may exert post-translational regulation of cyp7a enzyme activity. Treatment of C15.1 cells with HDL or LDL did not alter cyp7a enzyme activity, suggesting that lipoproteins do not modulate cyp7a enzyme activity post-translationally. Taken together, the results strongly suggest that the differences observed in conversion of HDL- and LDL-derived cholesterol to hydroxylated sterols is due to substrate availability to cyp7a and not to lipoprotein-mediated modulation of enzyme activity.

The results from the study demonstrate that cholesterol derived from HDL and LDL are delivered to different sub-cellular localizations withing the McA cells. Analysis of lipoprotein-derived cholesterol processing strongly suggests that LDL-derived cholesterol is more accessible to ACAT for esterification and that HDL-derived cholesterol is more accessible for cyp7a for conversion to bile acids and secretion as UC. Conversely, the secretion of cholesterol in LDL-treated cells was primarily in the form of CE. It is possible that this CE is secreted as an apoB-containing particle. The results suggest that while HDL-derived cholesterol may be destined for cholesterol elimination via bile acid production and biliary cholesterol secretion, LDL-derived cholesterol may be destined for lipoprotein secretion and re-circulation of cholesterol into the plasma. A model of the

metabolism of HDL- and LDL-derived cholesterol in McA cells is proposed in Figure 5-1.

5.2. Future Directions

The experiments undertaken in this thesis provide a better understanding of how lipoprotein cholesterol is metabolized in a liver-derived cell line and offers some insight as to how these processes occur in a hepatocyte. An unfortunate limitation of primary hepatocytes is that although possessing intact liver-specific functions, these abilities are lost over time (Follman 1990). Cyp7a gene expression and bile acid transport activity rapidly declines in culture hepatocytes in a time-span of approximately 24 hours (Torchia 1996), (Labonté 2000). Furthermore, to aid in the stabilization of liver-specific functions, hormones such as insulin are included in the culture medium (Laishes 1976). Insulin possesses the ability to activate signal transduction cascades (Cheatham 1992), (Shepherd 1998). In addition, cyp7a has been demonstrated to be responsive to regulation by insulin at the level of gene transcription (De Fabiani 2000), (Twisk 1995), (Crestani 1995), (Wang 1996). Thus, the use of primary hepatocytes in the time frame and conditions of the experiments designed is unfeasible.

Being an immortalized cell line, McA cells have lost several key cellular functions present in primary rat hepatocytes. McA cells have lost the ability to synthesize bile acids (Labonté 2000) and the ability to transport bile acids (Torchia 1996). Despite the loss of these functions, these deficiencies have

been exploited to better understand the nature of these missing functions. Stable expression of cyp7a has restored the bile acid biosynthetic pathway (Labonté 2000) and stable expression of the sodium/taurocholate co-transporting polypeptide restores bile acid transport in McA cells (Torchia 1996). The McA cell line thus provides an invaluable tool in which to characterize these and other liver-specific functions.

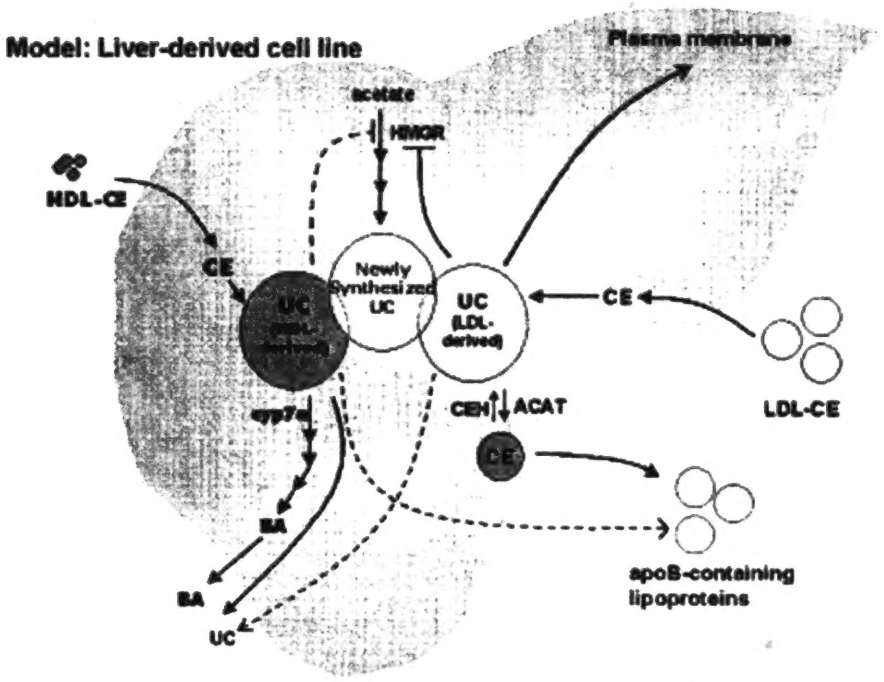


Figure 5-1. Proposed model illustrating the metabolism of HDL- and LDL-derived cholesterol in a liver-derived cell line.

Following internalization and hydrolysis, HDL- and LDL-derived cholesterol are delivered to pools distinct from each other. The HDL-derived UC pool is more accessible to cyp7a than LDL-derived UC. Conversely, LDL-derived cholesterol is delivered both to the plasma membrane and to a pool that is more accessible to ACAT than HDL-derived UC. After reaching their respective metabolic pools, HDL-derived UC is preferrentially utilized for bile acid and biliary cholesterol secretion, with a small proportion secreted as CE. LDL-derived UC is preferentially utilized for CE formation for lipoprotein assembly and secretion with a small proportion secreted as UC. Newly synthesized cholesterol provides substrate for both cyp7a and ACAT.

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Appendix I: Commonly used solutions/solvents

1. Filipin stain

Stock solution (5 ml)

Filipin complex	12.5 mg

Filipin complex was dissolved in dimethylformamide and stored protected from light at 4°C. Filipin stain was used at a 1:50 dilution in PBS (250 µg/ml).

2. Solvents for lipid extraction

Folch Lipid extraction	
Chloroform:Methanol	2:1 (v/v)
NaCl acidified with 2% acetic acid	1 M acidified with 2% acetic acid (v/v)
Bligh & Dyer Lipid extraction	
Methanol	
Chloroform	
NaCl acidified with 2% acetic acid	1 M acidified with 2% acetic acid (v/v)
Monophasic Lipid Extraction	
Hexane:Isopropanol,	3/2 (v/v)

3. Solvent systems for Thin-layer Chromatography

Solvent system for resolution of neutral lipids	
Hexane	70 ml
Diethyl Ether	30 ml
Methanol	5 ml
Glacial Acetic Acid	1 ml

Solvent system for resolution of 7α-hydroxycholesterol

Ethyl acetate	60 ml
Toluene	40 ml

4. Buffers for microsome preparation

Microsome Homogenization Buffer

KCl	50 mM
K_2PO_4, pH 7.4 (Mix of K_2HPO_4 and KH_2PO_4)	100 mM
Sucrose (make fresh)	300 mM
KF	50 mM
EDTA	1 mM
DTT (add fresh before using)	5 mM

2X CYP7 Assay Buffer

KCl	100 mM
K_2PO_4	200 mM
KF	100 mM
EDTA	2 mM
DTT (add fresh before using)	10 mM
(CHAPS	0.030%)

5. Buffer for Mammalian Cell Lysis

Lysis Buffer

Tris-HCl, pH 7.4	50 mM
NP-40	1%
Sodium Deoxycholate	0.25%
NaCl	150 mM

EGTA	1 mM
Complete™ Protease Inhibitor	¼ tablet per 10 ml

6. Buffers for sodium dodecyl sulfate (SDS)-Polyacrylamide Gel Electrophoresis

Stacking Buffer (for 500 ml)

Tris	1 M, pH 6.8

Tris was dissolved in deionized water, brought to pH 6.8 and filter sterilized.

Separating Buffer (for 500 ml)

Tris	1.5 M, pH 8.8

Tris was dissolved in deionized water, brought to pH 8.8 and filter sterilized.

2X Loading Buffer

Tris-HCl	100 mM
SDS	4%
Bromophenol Blue	0.2%
Glycerol	20%

Just prior to use, add 200 ul of 1 M dithiothreitol (DTT) to 800 ml of 2X Loading Buffer to make a final concentration of 200 mM DTT.

SDS-PAGE Running Buffer

Tris Base	25 mM
Glycine (electrophoresis grade)	250 mM
SDS	0.1% (w/v)

Combine and bring to 1 L final volume. Dilute to 1 X running buffer for running SDS-PAGE gels.

7. Buffers for Western Transfer

Electroblotting buffers

Buffer I

Tris	300 mM

Tris was dissolved in deionized water.

Buffer II

Tris	25 mM
Methanol	10% (v/v)

Tris and methanol were dissolved in deionized water.

Buffer III

Glycine	40 mM

Glycine was dissolved in Buffer II to a final concentration of 40 mM.

10 X Ponceau S (for 100 ml)

Ponceau S	2 g
Trichloroacetic acid	30 g
Sulfoacetic acid	30 g

Above was dissolved in deionized water and brought to a final volume of 100 ml. The solution was diluted 1:10 in deionized water for use.

20X PBS (for 4L)

NaCl	2.73 M
KCl	53.7 mM
Na_2HPO_4	60.3 mM
KH_2PO_4	29.4 mM

The above solutes were dissolved in deionized water, adjusted to pH 7.4 and brought to a final volume of 4L. Stock solution was diluted to 1X for use. The solution was stored at room temperature.

PBS-T

1X PBS	
Tween-20	0.1%

1 ml Tween-20 was mixed thoroughly into 1 L of 1X PBS. The solution was stored at room temperature.

Blocking Solution PBS-T+skim milk (for 1L)

Dried skim milk powder	5% (w/v)

Blocking solution was prepared by skim milk powder into previously prepared PBS-T. The solution was stored at 4°C and used immediately.

Enhanced Chemiluminescence (ECL) Solution

Tris-HCL, pH 8.5	12 ml
30% H_2O_2	3.8 µl
250 mM Luminol in dimethyl sulfoxide (DMSO)	60 µl
90 mM ρ-coumaric acid in DMSO	26.6 µl

Appendix II: Additional data for Chapter 2

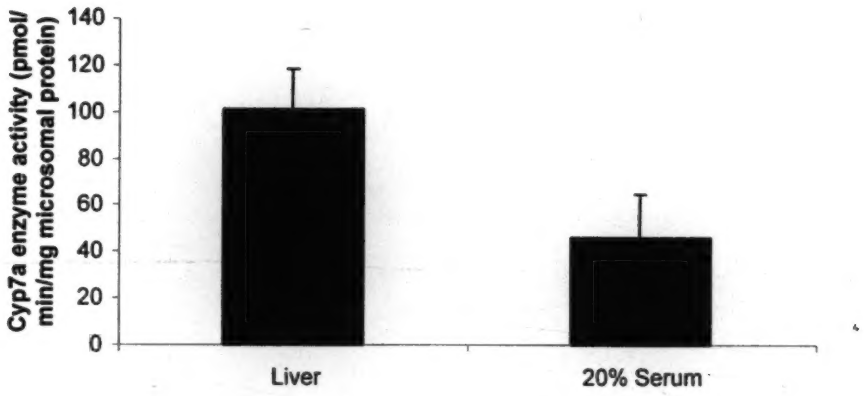

Figure II-A: Cyp7a enzyme activity in C15.1 cells.

Microsomes were prepared and cyp7a enzyme activity assayed as described in Section 2.9. C15.1 microsomal cyp7a enzyme activity is compared to that of liver microsomes from a mouse fed a diet containing 2% cholestyramine. Values shown represent ± S.D., *n*=3

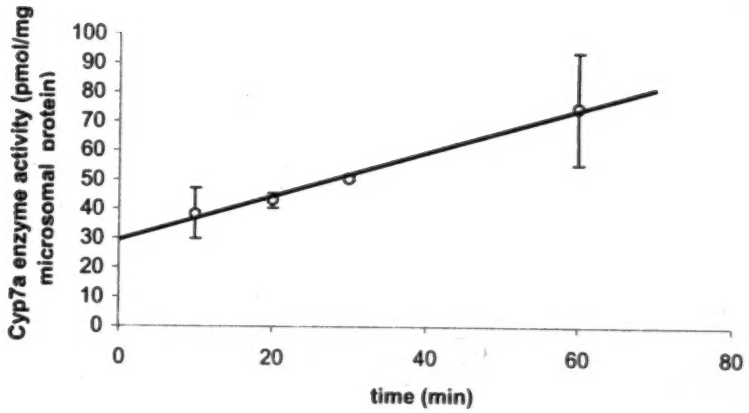

Figure II-B: Determination of incubation time for cyp7a assays.

Microsomes were prepared from C15.1 cells and cyp7a enzyme activity assayed as described in Section 2.9. Microsomes were incubated for 10-60 minutes with [^{14}C] cholesterol 2-hydroxypropyl-β-cyclodextrin complex. An incubation time of 30 minutes was chosen for all subsequent experiments. Data shown represent ± S.D., *n*=3.